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Prevalence of extended-spectrum β-lactamase-producing *Escherichia coli*, toxinogenic *Clostridium spp.* and *Yersinia enterocolitica* on Bavarian dairy and beef cattle farms

Inaugural-Dissertation zur Erlangung der tiermedizinischen Doktorwürde

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"The greatest obstacle to discovery is not ignorance, but the illusion of knowledge"

***(Daniel Boorstin*)**

TABLE OF CONTENT

ABBREVIATIONS

BfR	Bundesinstitut für Risikobewertung (Federal Institute for Risk Assessment)
bla	Gene encoding β-lactamase
BoNT	Botulinum Neurotoxin
CDC	Centers for Disease Control and Prevention
CDI	*C. difficile* infection
DANMAP	The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme
ECDC	European Center for Disease Prevention and Control
EFSA	European Food Safety Authority
e.g.	Example given
EHEC	Enterohemorrhagic *Escherichia coli*
ESBL	Extended-spectrum β-lactamase
EUROSTAT	Statistical office of the European Union
FVE	Federation of Veterinarians of Europe
i.e.	Id est (that is)
LPS	Lipopolysaccharides
PCR	Polymerase chain reaction
RKI	Robert Koch-Institut
USDA	United States Department of Agriculture

Abbreviations appearing in the two publications are explained there.

I. INTRODUCTION

“Food safety doesn't begin at the grocery store or in the kitchen. It begins on the farm.” (USDA, 2006)

Within Europe, concerns about food have largely shifted from ensuring an adequate supply of products towards food safety, animal and plant welfare, and traceability (EUROSTAT, 2008). To provide safe and healthy food the EU has adopted an integrated approach, from the farm to the fork, to ensure food safety and quality on each level of the food chain. “From farm to fork” aims at linking the entire chain of food production from raising animals and processing its products up to the retail level, assuring safe food being delivered to the consumer. This approach calls for risk assessment and surveillance on every level of the food chain as well as communication and interaction among all participants. At the level of primary production animal health and welfare are the main keys to public health, as they are linked to each other: Healthy and well-kept animals will produce healthy and safe products with, e.g. a highly reduced need for antibiotic treatment. In addition to healthy animals, epidemiologic surveillance systems on farms are necessary to prevent transmission of potential pathogens and resistance mechanisms via the food chain (FVE, 2012). Therefore, pathogen prevalence monitoring, as well as resistance monitoring, are an essential basis for risk assessment and rational discussions leading to actions that secure animal and public health equally.

Furthermore, resistance to clinically used antimicrobials is not a phenomenon that has come up during the last few years; it already emerged shortly after the first introduction of penicillin into clinical use in the 1940s. The number of resistant bacteria was low in these early days and therefore, was not considered as a significant problem. However, after the clinical introduction of new antimicrobial drugs, resistance could be detected shortly afterwards not only in pathogenic, but also in commensal bacteria (BARBOSA & LEVY, 2000; SCHWARZ et al., 2006).

The objective of this work was on one side to study the occurrence of extended-spectrum β-lactamase-producing *Escherichia coli* on dairy and beef cattle farms in Bavaria and to elucidate possible risk factors for their spread. It was conducted within the RESET joint research project (www.reset-verbund.de). This interdisciplinary research project, comprising federal and regional authorities, as well as universities, was funded by the Ministry of Education and Research (Bundesministerium für Bildung und Forschung) and collects data on the epidemiology and genetics of ESBL *E. coli* in different fields of research (veterinary and human medicine, the environment and food) to determine the origin, way of transmission and the risk for humans being exposed to them.

On the other side the occurrence of potentially foodborne pathogens, namely *Clostridium difficile*, *Clostridium botulinum* and *Yersinia enterocolitica* was assessed in healthy cattle of different ages and production types to estimate the potential risk of transmitting these bacteria via the food chain. There is a paucity of studies concerning prevalence rates of both parts of this work in Germany and other European countries, particularly on a country-wide or regional scale. This would be necessary to identify dynamics and risk factors for transmitting potentially zoonotic pathogens and resistance mechanisms from animals to humans.

II. LITERATURE OVERVIEW

1. Extended-spectrum β-lactamase-producing *Escherichia coli*

1.1. *Escherichia coli* – general information

Escherichia (*E.*) *coli* is a gram-negative, rod-shaped bacterium, 2 to 6 µm in length belonging to the family *Enterobacteriaceae*. Most strains are motile due to peritrichous flagella and fimbriae. *E. coli* are oxidase-negative, catalase-positive, non-spore forming facultative anaerobes which ferment lactose and produce indole (SONGER et al., 2005). However, there exist some strains that do not ferment lactose. *E. coli* grow well on non-selective media and certain strains show hemolytic activity on blood agar. On MacConkey agar *E. coli* grow as they are not inhibited by the bile salts in the medium. Because of the acid production from lactose fermentation colonies on MacConkey agar and the surrounding medium are pink (QUINN et al., 2001). To distinguish *E. coli* from other members of the *Enterobacteriaceae* family the growth characteristics and biochemical reactions as described above are very important. Furthermore, *E. coli* isolates and isolates of *Enterobacteriaceae* in general are serotyped using somatic (O), flagellar (H) and capsular (K) antigens. Somatic antigens are lipopolysaccharides which are located at the surface of the cell wall. Flagellar antigens are proteins and capsular antigens consist of polysaccharides (QUINN et al., 2001).

Shortly after birth the intestinal tract of humans and a multitude of animals is colonized by *E. coli* which are part of the normal flora of the large intestine. However, not all *E. coli* strains are commensal and non-pathogenic: Pathogenic strains possess virulence factors including capsular polysaccharides which interfere with the complement system, fimbrial adhesins, intimin (another adhesin), enterotoxins, cytotoxic necrotizing factors, cytolethal distending factors and hemolysin (QUINN et al., 2001).

Pathogenic *E. coli* can be categorized into pathotypes: Six pathotypes are associated with diarrhea and collectively are referred to as diarrheagenic *E. coli*:

- Enterohemorrhagic *E. coli* (EHEC)
- Enterotoxigenic *E*. *coli* (ETEC)
- Enteropathogenic *E*. *coli* (EPEC)
- Enteroaggregative *E*. *coli* (EAEC)
- Enteroinvasive *E*. *coli* (EIEC)
- Diffusely adherent *E. coli* (DAEC)

Besides all six pathotypes, EHEC is one of the most important ones and commonly associated with foodborne outbreaks (CDC, 2012).

1.2. Extended-spectrum β-lactamase

Extended-spectrum β-lactamases (ESBLs) are enzymes that are able to hydrolyze nearly all β-lactam antibiotics, such as penicillins, oxyminocephalosporins, monobactams and cephalosporins up to generation 3 and 4, but usually not carbapenems and cephamycins. They are commonly inhibited by β-lactamase inhibitors as clavulanic acid, sulbactam and tazobactam. ESBLs are distributed worldwide and have been detected in various bacteria belonging to the family *Enterobacteriaceae* as *Klebsiella pneumoniae, E. coli, Proteus mirabilis* and *Salmonella* species. The enzymes are plasmid-encoded which facilitates gene transfer between bacteria from the same species or even family and therefore, fast and efficient spread of resistance (BONNET, 2004). The most commonly found ESBLs harbor genes belonging to the TEM, SHV and CTX-M families.

Classification

1.2.1.1. Ambler/Bush classification

β-lactamases are classified into four classes (Ambler class A-D) with regard to their molecular structure: class A, C and D containing serine β-lactamases and class B metallo-β-lactamases. They are further divided into four groups (Bush-Jacoby group 1-4) and several subgroups defined by their substrate and inhibitor profiles (BUSH & JACOBY, 2010):

Group 1: Cephalosporinases that are not well inhibited by clavulanic acid

Group 2: Penicillinases that are generally inhibited by active-site directed β-lactamase inhibitors

Group 2be: Penicillinases that have extended spectrum of activity

Group 3: Metallo-β-lactamases that are poorly inhibited by all classical β-lactamase inhibitors except EDTA

Group 4: Are omitted in the present scheme as they have been characterized incompletely.

TABLE 1. Classification schemes for bacterial β-lactamases from BUSH & JACOBY (2010).

Bush-Jacoby group	Ambler Class	Distinctive substrate(s)	Inhibited by CA[1] or TZB[2]	Representative enzymes
1	C	Cephalosporins	No	CMY-2, FOX-1, MIR-1
1e	C	Cephalosporins	No	GC1, CMY-37
2a	A	Penicillins	Yes	PC1
2b	A	Penicillins, early cephalosporins	Yes	TEM-1, TEM-2, SHV-1
2be	A	Extended-spectrum cephalosporins, monobactams	Yes	TEM-3, SHV-2, CTX-M-15

TABLE 1. Classification schemes for bacterial β-lactamases from BUSH & JACOBY (2010).

Bush-Jacoby group	Ambler Class	Distinctive substrate(s)	Inhibited by CA[1] or TZB[2]	Representative enzymes
2br	A	Penicillins	No	TEM-30, SHV-10
2ber	A	Extended-spectrum cephalosporins, monobactams	No	TEM-50
2c	A	Carbenicillin	Yes	PSE-1, CARB-3
2ce	A	Carbenicillin, cefepime	Yes	RTG-4
2d	D	Cloxacillin	Variable	OXA-1, OXA-10
2de	D	Extended-spectrum cephalosporins	Variable	OXA-11, OXA-15
2df	D	Carbapenems	Variable	OXA-23, OXA-48
2e	A	Extended-spectrum cephalosporins	Yes	CepA
2f	A	Carbapenems	Variable	KPC-2, IMI-1, SME-1
3a	B (B1)	Carbapenems	No	IMP-1, VIM-1, CcrA, IND-1
3b	B (B2)	Carbapenems	No	CphA, Sfh-1
NI[3]	4	Unknown		

[1]CA: clavulanic acid;

[2]TZB: tazobactam;

[3]NI: not included.

Common ESBLs as TEM, SHV and CTX-M belong to Ambler Class A and Bush-Jacoby group 2be. Chromosomal- or plasmid-encoded AmpC β-lactamases (see 1.2.1.5) are classified as Ambler class C and Bush-Jacoby group 1 and OXA-type ESBLs belong to Ambler Class D (Table 1).

1.2.1.2. TEM, SHV

Broad-spectrum TEM β-lactamases were biochemically characterized in 1966 on a strain isolated from a patient in Greece. This β-lactamase was plasmid-mediated and able to hydrolyze ampicillin and related antimicrobials as piperacillin and carbenicillin. Due to point mutation TEM ESBLs evolved from the broad spectrum TEM-1 and -2 genes and are now able to hydrolyze 3rd and 4th generation cephalosporins (PFEIFER et al., 2010). The point mutations lead to 1-4 amino acid substitutions which induce changes in the active site of the enzyme reducing activity of 3rd and 4th generation cephalosporins and monobactams (WINOKUR et al., 2001).

SHV ESBLs evolved in a similar way to TEM ESBLs by point mutation of the broad spectrum SHV-1 genes. SHV enzymes have evolved from chromosomal *Klebsiella pneumoniae* specific β-lactamases which were then mobilized onto plasmids (HAEGGMAN et al., 1997).

Until the 2000s TEM and SHV-derived ESBLs were the predominant variants found most frequently in *Klebsiella spp.*, but also in *E. coli* and other members of the *Enterobacteriaceae* family causing nosocomial infections (EFSA, 2011).

1.2.1.3. OXA

Most OXA-type ESBL enzymes (OXA-11,-14 and -16) derive from point mutations from the classical plasmid-mediated OXA-10 or from OXA-15 (OXA-19 and -28) which are resistant to penicillins and 3rd generation cephalosporins (PFEIFER et al., 2010). These OXA-type ESBLs were predominantly found in *Pseudomonas aeruginosa* and only sporadically in *Enterobacteriaceae* (BRADFORD et al., 1999). However, a number of different class D OXA enzymes, as OXA-48, show resistance to carbapenems and were not only reported from *P. aeruginosa* but also from *Enterobacteriaceae* (PFEIFER et al., 2010).

1.2.1.4. CTX-M

Since the early 2000s CTX-M genes, which are genetically unrelated to TEM or SHV, emerged in human and animal isolates. Since then, CTX-M genes have emerged and spread worldwide with at present 148 molecular variants belonging to the CTX-M family (JACOBY, 2013). In 1989 BAUERNFEIND et al. (1990) first described a new extended-spectrum β-lactamase in an *E. coli* isolate from ear exudate of a newborn in Munich. It was plasmid-borne and able to hydrolyze cefotaxime at a higher rate than ceftazidime, which was unique compared to other ESBLs, and was therefore called CTX-M (CTX for cefotaximase, -M for Munich). However, newer CTX-M variants are able to hydrolyze ceftazidime as well due to mutations.

CTX-M β-lactamases are divided into five clusters considering amino acid sequence similarities:

CTX-M group 1: main members are CTX-M-1,-3,-10,-11,-12,-15,-22,-23,-28,-29,-30,-32,-33,-36,-54,-58,-66,-79,-116

CTX-M group 2: main members are CTX-M-2,-4,-6,-7,-20,-31,-35,-42,-44

CTX-M group 8: main members are CTX-M-8,-40,-63

CTX-M group 9: main members are CTX-M-9,-13,-14,-16,-17,-18,-19,-24,-27,-45,-46,-47,-48,-49,-50,-65,-104

CTX-M group 25: main members are CTX-M-25,-26,-39,-41,

(CANTON & COQUE, 2006; PFEIFER et al., 2010; CANTON et al., 2012)

Phylogenetic analyses suggest that CTX-Ms did not originate by point mutations from plasmid mediated enzymes as TEM and SHV, but through mobilization of chromosomal β-lactamase genes from different environmental *Kluyvera* (*K*.) species onto plasmids (CANTON et al., 2012). *K. ascorbata* is thought to be the progenitor of CTX-M-1 and -2 group enzymes (HUMENIUK et al., 2002) and some of the CTX-M-1 group β-lactamase genes seem to originate from *K. cryocrescens* (DECOUSSER et al., 2001). The progenitor of CTX-M-8 and -9 enzymes is thought to be from *K. georgiana* (POIREL et al., 2002).

ESBL resistance genes and resistance determinants against aminoglycosides, tetracycline, fluoroquinolones and sulfonamids are often situated on the same plasmid (JACOBY & SUTTON, 1991) and therefore, the use of any of these antimicrobials can co-select for all other ones. Plasmids that encode antimicrobial resistance genes can also code for genes mediating resistance against disinfectants, heavy metal tolerance, virulence and metabolic functions and therefore, could be co-selected (BARBOSA & LEVY, 2000) as well.

1.2.1.5. AmpC

The phenotypic resistance patterns are similar to ESBLs, as AmpC β-lactamases are able to hydrolyze penicillins and cephalosporins, including oxymino-cephalosporins and monobactams. Moreover, they are able to hydrolyze cephamycins. AmpC β-lactamases however, are not inhibited by clavulanate, sulbactam and tazobactam (LI et al., 2007). Resistance can be mediated by hyperproduction of chromosomal AmpC β-lactamases which is caused by promoter mutations increasing the transcription rate (CAROFF et al., 2000), or can be due to plasmidic AmpC enzymes. Plasmidic enzymes belong to Ambler Class C and fall into six phylogenetic groups, CMY-2 being the most predominant one (EFSA, 2011).

1.3. Epidemiology

1.3.1. Human hosts

To assess the spread of ESBLs in humans, animals and food, data about the prevalence in different hosts is needed. However to date, there is still a lack of studies on a country-wide scale as most reports describe only circumscribed areas. Furthermore harmonized methodologies used in the studies would make it easier to compare prevalence rates from different countries as the use of selective media for the detection of ESBL increases detection rate and therefore, prevalence.

Another important fact is that especially in humans, prevalence rates of ESBL *E. coli* are mostly reported from clinical isolates from hospitals or the community. As *E. coli* are not only pathogenic bacteria but also commensal it would be necessary to test healthy people to determine the real prevalence of ESBL *E. coli* in humans. However, these studies are scarce (PFEIFER et al., 2013).

A significant increase in ESBL-producing bacteria was detected in the EU for the period 2008-2011 with great variations in the occurrence and distribution among different countries: *E. coli* isolates resistant to 3rd generation cephalosporins were reported from 29 European countries with prevalences ranging from 3% in Sweden up to 36.2% in Cyprus (Figure 1) (ECDC, 2012). Among these resistant isolates between 85% and 100% were reported to be ESBL positive. In Germany occurrence of resistance to 3rd generation cephalosporins in *E. coli* increased from 1.7% in 2005 to 8% in 2011 (ECDC, 2012).
In the United States increasing numbers of *E. coli* resistant to 3rd generation cephalosporins were reported by the MYSTIC program (RHOMBERG & JONES, 2009) with 0.5% in 1999 to 5.1% in 2008. Another publication with data from the same surveillance program (GOOSSENS & GRABEIN, 2005) determined the prevalence of ESBL phenotypes in *E. coli* in Europe and the U.S for the year 2004: In Europe 10.8% of all *E. coli* isolates were described as ESBL-producing in contrast to 1.4% in the U.S.
In the Asia-Pacific region the total prevalence of phenotypic ESBL *E. coli* was described to be 7.5% in the SMART study in 2010 (HSUEH, 2012). This study further reported occurrence rates of *E. coli* resistant to 3rd generation cephalosporins from 21.3% in China up to 61.5% in Singapore for the same year. In Latin America ESBL phenotype rates in *E. coli* ranged from 12.8% in Brazil up to 48.8% in Mexico for the years 2008-2010 (GALES et al., 2012).

In most European countries ESBL-producing *E. coli* are recognized to be endemic with a dominance of CTX-M enzymes. In other countries, such as the USA, CTX-M-producing *E. coli* have only been sporadically reported (Figure 2).

In the U.S. detection of ESBLs is less frequent, however, CMY-like β-lactamases are reported more often there than in Europe (DOI et al., 2010).

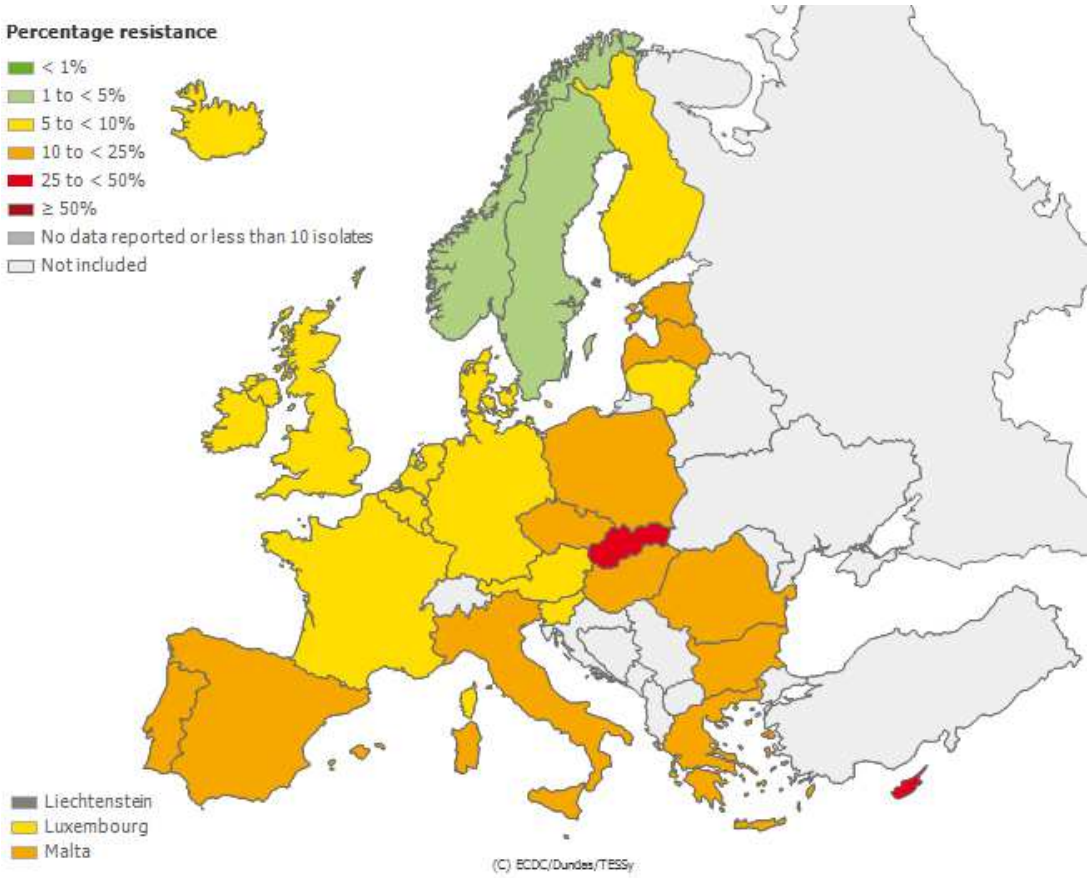

FIGURE 1. Percentage (%) of invasive *E. coli* isolates with resistance to third-generation cephalosporins by country, 2011 (ECDC, 2012).

ESBLs are distributed worldwide inequally with some CTX-M enzymes being well represented in circumscribed geographic regions: In Northern European countries a dominance of CTX-M and SHV enzymes is reported, with CTX-M-1 being the predominant group followed by CTX-M-9-like enzymes. High prevalence of CTX-M-9 group enzymes were described in the countries surrounding the Mediterranean Sea and in the United Kingdom (UK) as well. Furthermore, in Southern European countries and the UK CTX-M-1, CTX-M-14, and to a lesser extent SHV and TEM enzymes are represented. CTX-M-3 has been mainly described in Eastern European countries, where SHV and

TEM were sporadically found, too. In South America and Japan CTX-M-2 enzymes are the predominant ESBLs and in Belarus and Russia CTX-M-5. CTX-M-15 is spread nearly worldwide among humans (COQUE et al., 2008).

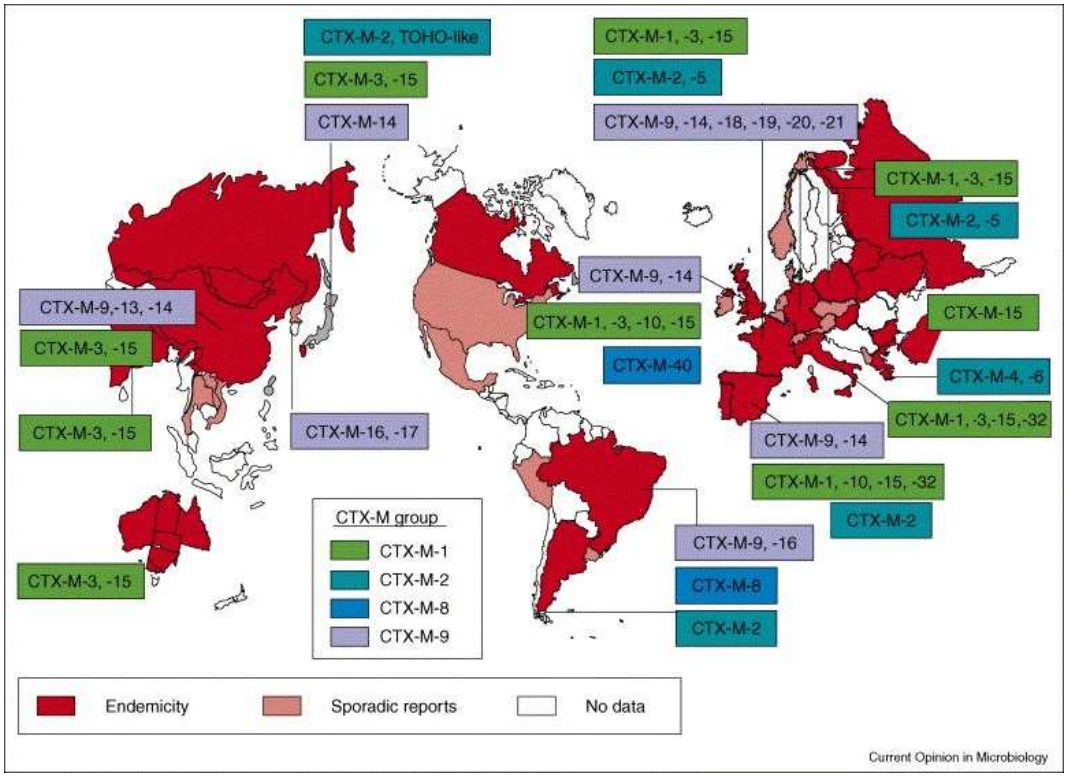

FIGURE 2. Geographic distribution of ESBL resistance genes in humans [altered from CANTON & COQUE (2006)].

1.3.2. Animal hosts

ESBL *E. coli* has been detected in a broad range of different animals including food-producing animals (BRINAS et al., 2003; MEUNIER et al., 2006; HORTON et al., 2011), pets (COSTA et al., 2004; CARATTOLI et al., 2005) and wild animals (POETA et al., 2009; BONNEDAHL et al., 2010; RADHOUANI et al., 2013). However, as described above, different methods can make it difficult to compare prevalence rates from various studies.

The occurrence of ESBL *E. coli* in poultry ranged from 10.7% in France, to 54.5% of broilers in Poland, 63.4% of chickens at the slaughterhouse in Switzerland and up to 79.9% of poultry tested in Spain (BLANC et al., 2006). In a Dutch study (DIERIKX et al., 2013) 85% of chicken farms had a within-flock prevalence of 80% and first results of a German study which was

conducted within the RESET joint research project reported 74% of tested fecal samples from broilers harboring phenotypic ESBL *E. coli* (HERING, 2012).

In pigs prevalence of ESBL *E. coli* ranged from 12% in 2010 to 4.5% in 2011 in Denmark, 15.3% in Switzerland, 33.3% in Poland and up to 100% in a Spanish study (BLANC et al., 2006). The German study described above (HERING, 2012) reported 68% of tested pigs shedding phenotypic ESBL *E. coli*.

Prevalence of ESBL *E. coli* in cattle was reported to be 13.7% in Switzerland (GESER et al., 2012), 4.1% in France (MADEC et al., 2008), 44.3% in Great Britain (HORTON et al., 2011) and 10.2% in Denmark (DANMAP, 2012). In a study carried out in southern Bavaria ESBL *E. coli* could be detected in 32.8% of samples originating from cattle and their environment (SCHMID et al., 2013).

Similar as in humans, ESBLs are spread differently among food animals and food in European countries: CTX-M-1 is predominantly reported in most European countries. CTX-M-14 and CTX-M-32 enzymes are more commonly found in countries surrounding the Mediterranean Sea and Southern European countries, whereas CTX-M-14 was found in food animals in the UK and Belgium, too. In Central and Northern European countries, including the UK and Ireland, CTX-M-2 was detected as well. However, in contrast to human ESBLs CTX-M-15 was only sporadically reported in animals. Furthermore, SHV- and TEM-producing isolates were detected in food-producing animals and food, especially SHV-12, SHV-2 and TEM-52 (EFSA, 2011).

1.3.3. Food from animal origin

ESBL *E. coli* were not only reported in food-producing animals but also in their products: In Germany cefotaxime resistant *E. coli* were detected in laying hens, broilers, turkeys, pork, veal, and bulk tank milk. However, highest prevalence rates were found in broilers in 2010 with 13.5% of all *E. coli* isolates being resistant against cefotaxime followed by veal with 10.3% (BFR, 2011). Detection rates for other food products originating from animals ranged from 0-3.2% (Table 2). In Denmark ESBL *E. coli* were commonly isolated from 44% of

broiler meat in 2011 and very rarely in pork and beef (0-0.9%) (DANMAP, 2012). In contrast to other European countries Sweden has not detected any ESBL *E. coli* in pork, however, 44% of broiler meat samples were positive. Reports from the Netherlands show that 84-100% of poultry meat was positive for ESBL.

TABLE 2. Percentage of *E. coli* isolates resistant against cefotaxime obtained from various animals and matrices in 2010 (BFR, 2011).

	Laying hens	Broilers	Turkeys	Turkey meat	Veal calves	Bulk tank milk
E. coli isolates	27/1001 (2.7%)	27/200 (13.5%)	0/127 (0%)	8/356 (2.2%)	28/272 (10.3%)	3/95 (3.2%)

1.4. Transmission

ESBLs have been detected in humans, in a variety of animal species and they could also be found in food products. Studies show that commensal *E. coli* can be a reservoir for similar resistance genes in human and animal hosts: LEVERSTEIN-VAN HALL et al. (2011) screened samples from poultry, retail chicken meat and humans for the presence of ESBL *E. coli* and the isolates were further analyzed to detect a potential genetic relationship between human and animal isolates. This study reported that ESBL genes, located on plasmids, were genetically indistinguishable from human and poultry *E. coli* isolates. Most of these genes were CTX-M-1 and TEM-52 producers with indistinguishable plasmids and isolate genotypes. These findings suggest that a transmission of ESBLs via the food chain may potentially occur.

1.5. Public health consequences

For many years the public health consequences of antimicrobial resistant bacteria transmitted from food animals to humans have been discussed. Consequences include reduced efficacy of early empirical treatment, limitations in the choices for treatment after microbiological testing, leading to more frequent treatment failures. Furthermore, this can result in increased severity of infection, increased duration of illness, and increased risk of hospitalization or mortality (ANGULO et al., 2004; MOLBAK, 2004).

Humans themselves can be a source of ESBL producing bacteria especially in hospitals: ESBLs are usually more prevalent in wards with hospitalized patients at high risk, i.e. in intensive care units, surgical wards, pediatrics and neonatology wards (GNIADKOWSKI, 2001). Resistant bacteria can be transmitted from patient to patient and it may also be imported from a new patient entering the hospital, leading to nosocomial infections. In 2011 a nosocomial outbreak of ESBL producing *Klebsiella pneumoniae* took place in a neonatology ward of a hospital in Bremen, Germany. In 25 children *K. pneumoniae* could be detected, nine children developed a sepsis and four eventually died (STAUCH, 2011).

Another consequence for public health is that commensal *E. coli* can be a reservoir for ESBL resistance genes and can potentially pass these genes to pathogenic bacteria as e.g. *Salmonella spp.* (WINOKUR et al., 2001). The only approved treatment of *Salmonella* infections in children is ceftriaxone which is a third generation cephalosporin, similar to ceftiofur, used as treatment of companion and food animals. Ceftriaxone is further used in the treatment of fluoroquinolone-resistant salmonellosis in adults (DUNNE et al., 2000). Scientists are discussing a potential occurrence of cross resistance among these two antimicrobials leading to treatment failures of *Salmonella* infections in humans.

Furthermore, ESBL resistance genes are often located on the same plasmids as resistance determinants against aminoglycosides, tetracycline and

sulfonamids (JACOBY & SUTTON, 1991). These plasmids can also carry genes mediating resistance against disinfectants, heavy metal tolerance, virulence and metabolic functions (BARBOSA & LEVY, 2000). Due to the coexistence of multiple resistance determinants on a single plasmid the use of one antimicrobial can result in selecting resistance to all other antimicrobials.

II. Clostridium difficile

2.1. *Clostridium difficile* - general information

Clostridium difficile (*C. difficile*) are strictly anaerobic, gram-positive bacilli measuring 2.5-5.9 x 0.3-1.5 µm. The shape varies from very short to large rods which can produce subterminal endospores. The organisms are motile due to peritrichous flagella and they are both catalase- and oxidase-negative (HAFIZ & OAKLEY, 1976).

The virulence of *C. difficile* is mainly related to the production of two protein exotoxins, toxin A and toxin B, encoded by the *tcdA* and *tcdB* genes. Toxin A is an inflammatory enterotoxin that possesses weak cytotoxic activity, in contrast to toxin B, which is a very potent cytotoxin (KYNE et al., 2001). Both toxins cause increased vascular permeability and hemorrhage, however, toxin A can further cause fluid accumulation shown in various animal models (LYERLY et al., 1985). Some strains have an additional binary toxin (CDT) (POPOFF et al., 1988), encoded by *cdtA* and *cdtB* genes, which has cytotoxic and enterotoxic activities (CARMAN et al., 2011).

2.2 Epidemiology

2.2.1. *C. difficile* infection in humans

C. difficile infection (CDI) in humans can range from asymptomatic carriage to uncomplicated diarrhea up to severe and life-threatening pseudomembranous colitis often with systemic symptoms, i.e. fever, nausea, anorexia, malaise and dehydration (KELLY et al., 1994). CDIs are usually nosocomial infections and most cases have been documented in the hospital environment.

The main risk factors for acquiring a CDI are use of antimicrobials, hospitalization, severity of underlying disease and an age > 65 years. Due to antibiotic therapy the normal colonic microbiota is disturbed, ingested spores can germinate and *C. difficile* can colonize the colon, producing toxins. This can result in an asymptomatic carriage and contamination of the hospital environment or in diarrhea and colitis (KYNE et al., 2001). However, surveys in postnatal wards show that neonates are often transiently colonized by toxigenic *C. difficile* without developing disease (LARSON et al., 1982). A study performed by EGLOW et al. (1992) suggests that this resistance in neonates could be due to immature toxin receptors.

Recently, an increase of incidence and severity has been reported in hospitals in the U.S. where the diagnosis of CDI has been doubled from 31 per 100,000 people in 1996 to 61 per 100.000 in 2003 (MCDONALD et al., 2006). This increase seems to be related to the emergence of a new, hypervirulent strain, namely PCR ribotype 027. Ribotype 027 is characterized as toxinotype III, North American pulsed field gel electrophoresis type 1 (NAP1) and carries genes for toxin A and B and the binary toxin (LOO et al., 2005; MCDONALD et al., 2005). The increase of virulence appears to be due to the hyperproduction of toxins (WARNY et al., 2005). This strain has not only emerged in Northern America but also in Japan and Europe where CDI has been associated with increased severity, high relapse rate and significant mortality (Figure 3).

FIGURE 3. Distribution of *C. difficile* ribotype 027 in Europe as of June 2008 (KUIJPER et al., 2008).

Furthermore, another new strain, namely PCR ribotype 078, has been described to be one of the most prevalent strains in Europe (BAUER et al., 2011). Ribotype 078 is characterized as toxinotype V and contains genes for toxins A and B as well as the binary toxin. In contrast to type 027, 078 affects a younger population with similar severity and is more frequently community associated (GOORHUIS et al., 2008). The main PCR ribotypes found in human CDI cases in Germany are 001, followed by 078 and 027 as shown in Figure 4.

Recently published data from the U.S. reported an increase of CDI in non-hospitalized patients (KHANNA et al., 2012). This study showed that 41% of 385 CDI cases were community-associated affecting people that have previously been thought to be at low risk, as young adults and children without any exposure to antimicrobials or hospitals.

2.2.2. *C. difficile* infection in animals

C. difficile has been detected in fecal samples from a variety of healthy and sick animals, however to date, their definite significance as a primary pathogen has not yet been clearly identified for all animal species.
C. difficile was documented as a major cause of enteritis in neonatal pigs which start scouring shortly after birth, sometimes with dyspnea, mild abdominal distension and scrotal edema and commonly with yellow, pasty diarrhea (WATERS et al., 1998; SONGER et al., 2000).

In adult horses *C. difficile* was associated with antibiotic-associated diarrhea but it could also be isolated from untreated diarrheic horses (BAVERUD et al., 2003). Furthermore, the study showed, that *C. difficile* could also be isolated from one in three normal healthy foals < 14 days of age. WEESE et al. (2001) reported that *C. difficile* was isolated from 12.7% of horses and 35.5% of foals with colitis.

Poultry is the food animal species which has been less investigated concerning the occurrence of *C. difficile* in general and its role as a primary pathogen. There are no studies about clinical symptoms in poultry related to *C. difficile* and only a few which detected it in healthy poultry (SIMANGO, 2006; INDRA et al., 2009; KOENE et al., 2012).
In cattle *C. difficile* was suggested to be associated with calf diarrhea as toxins could be detected more often in diarrheic than in non-diarrheic calves (RODRIGUEZ-PALACIOS et al., 2006). The same authors conducted another study where calves were orally inoculated with *C. difficile (*RODRIGUEZ-PALACIOS et al., 2007): The organism could be cultured from seven of eight inoculated calves and from none of the control calves, but no detection of

toxins or induction of enteric disease was possible. Furthermore, five of six control calves developed diarrhea and only two of the inoculated ones, which questions the role of *C. difficile* as a primary pathogen in calves.

C. difficile has been reported from healthy and sick dogs and cats in Germany (WEBER et al., 1989) however, these authors did not suggest that *C. difficile* is a significant pathogen for enteritis in pets. Another study showed that shedding of *C. difficile* in dogs is not correlated with clinical diarrheal signs even among toxigenic strains (STRUBLE et al., 1994; WEESE et al., 2010a).

2.3. Public health consequences

Public health consequences have been widely discussed for *C. difficile*, however, no transmission from animals or food to humans has yet been documented. Various authors suggest a possible spread of *C. difficile* from animals to humans, not only limited to food animals (ARROYO et al., 2005) but also from companion animals (BAVERUD et al., 2003; WEESE et al., 2010a). The organism could be found in various sick animals as well as in healthy ones, shedding *C. difficile* into the environment and therefore, being a potential reservoir.

Furthermore, *C. difficile* was not only isolated from food animals but also from their products: Various studies isolated it from ground beef and veal with prevalence rates ranging from 0% in Austria and The Netherlands (INDRA et al., 2009; DE BOER et al., 2011) up to 50% in the U.S. (SONGER et al., 2009). The same study further revealed that *C. difficile* was found in pork and turkey products as well as in ready-to-eat products. Moreover, raw vegetables, and samples from the environment, i.e. soil, river, sea and tab water, were found to harbor *C. difficile* (AL SAIF & BRAZIER, 1996).
These findings suggest that animals, food and the environment can be a potential reservoir for *C. difficile*, however, to assess a possible spread genetic relatedness has to be evaluated: In calves 94% of all strains and in pigs 83% belonged to PCR ribotype 078 (KEEL et al., 2007) and strains obtained from

meat samples (SONGER et al., 2009; WEESE et al., 2010b) shared the same ribotype which is one of the most prevalent strains in Europe (Figure 4).

KOENE et al. (2012) reported a genotypic relation of some porcine and human ribotype 078 strains by multiple locus variable-number tandem-repeat analysis (MLVA). However, they also concluded that most strains are predominantly animal host specific, whereas some strains share ribotypes among different animal species, as well (KOENE et al., 2012). Another Dutch study (DEBAST et al., 2009) identified a clonal complex, containing porcine and human ribotype 078 strains which showed a similar antimicrobial susceptibility profile and concluded that these strains were indistinguishable.

Although existing studies report of genetically closely related strains among animals and humans the actual transmission of *C. difficile* to humans has never been proved, therefore it is still unclear if *C. difficile* is a potential foodborne agent.

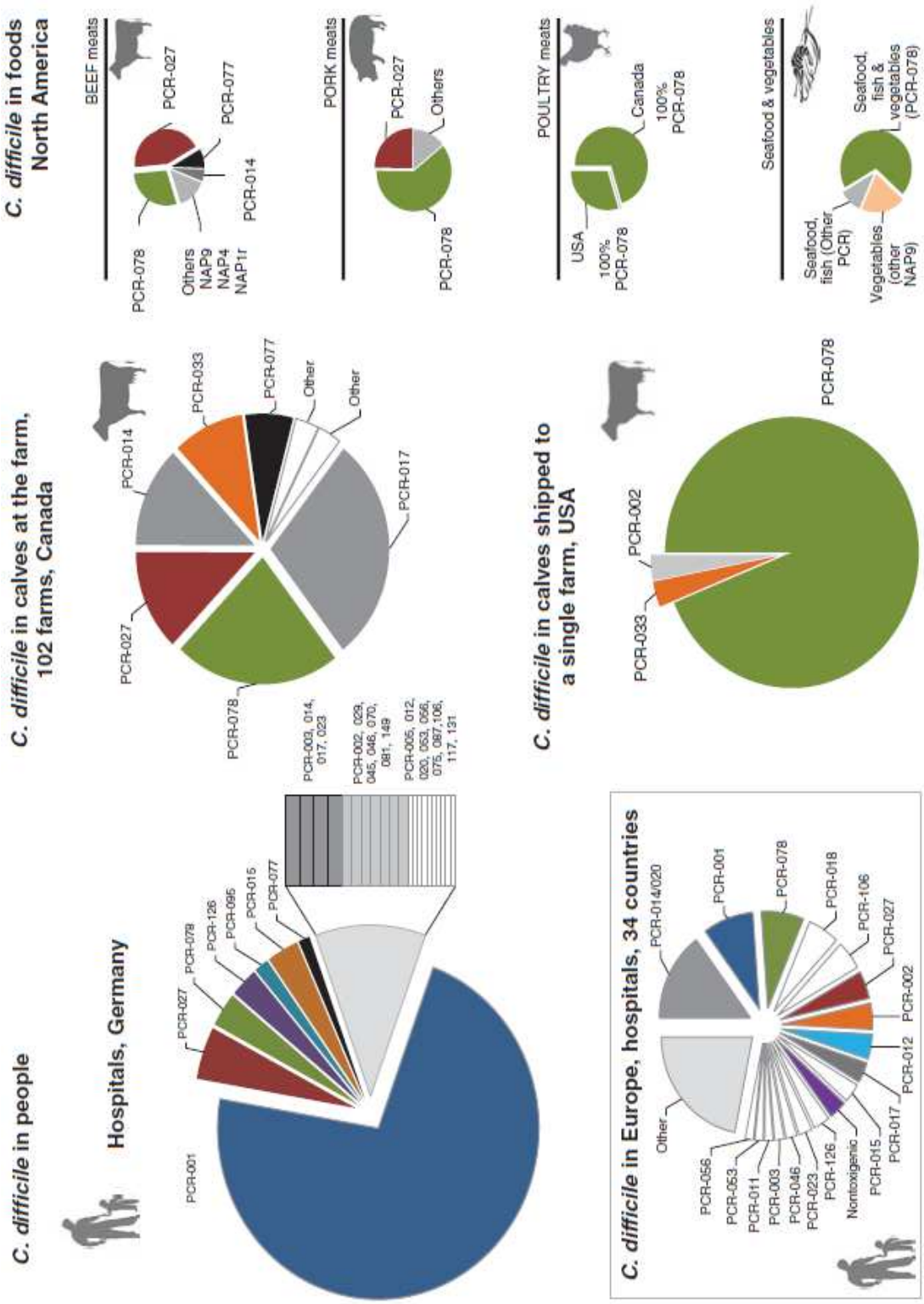

FIGURE 4. Frequency of isolation of toxigenic *C. difficile* PCR ribotypes from humans, young cattle, and various foods (RODRIGUEZ-PALACIOS et al., 2013).

3. *Clostridium botulinum*

3.1. *Clostridium botulinum* – general information

Clostridium (C.) botulinum is a large, gram-positive, anaerobic, rod-shaped bacterium which produces subterminal endospores. The endospores are distributed in soils and aquatic environments worldwide and germination of them occur in anaerobic locations as in decaying organic matter or in contaminated cans of meat or vegetables (QUINN et al., 2001).

C. botulinum produces eight neurotoxins (BoNT), namely types A, B, C_1, C_2, D, E, F, and G. They are among the most toxic substances known (mouse LD_{50}<0.1ng/kg). Types A, B, E, and very rarely F cause human botulism outbreaks, whereas types C and D are linked with most botulism outbreaks in animals. There is no evidence that type G (now *C. argentinense*) can cause disease (DODDS & AUSTIN, 2001). BoNTs A, B, E, and F are encoded by genes on the bacterial chromosome, in contrast to C and D where genes are encoded by bacteriophages and genes for BoNT G are located on a plasmid (DODDS & AUSTIN, 2001). Furthermore, *C. botulinum* is divided into four groups based on physiological differences as shown in Table 3.

TABLE 3. Grouping of strains of *C. botulinum* altered from DODDS & AUSTIN (2001).

Characteristics	I	II	III	IV
Neurotoxin type(s)	A, B, F	B, E, F	C, D	G (now *C. argentinense*)
Proteolysis	+	-	-	-
Outbreaks	Humans, animals	Humans, animals	Animals	-
Toxin gene	Chromosome	Chromosome	Bacteriophage	Plasmid

3.2. Epidemiology

3.2.1. *C. botulinum* infection in humans

Human botulism can be divided into five categories (DODDS & AUSTIN, 2001; SOBEL et al., 2005):

a) Foodborne botulism

Foodborne botulism is an intoxication which is caused by consumption of food contaminated with preformed BoNT. The toxins are absorbed from the gastrointestinal tract and reach peripheral motor nerve terminals via the blood stream. There, they block neurotransmission by inhibiting acetylcholine release which results in flaccid paralysis (DODDS & AUSTIN, 2001).

b) Infant botulism

This type of botulism is caused by ingestion of spores which can germinate and colonize the intestinal tract of infants less than one year of age, as the normal intestinal flora has not yet been fully established. Subsequently, neurotoxins are produced which are then absorbed and result in flaccid paralysis. This is the most common form of botulism in the United States (CDC, 2010).

c) Infectious botulism

Infectious botulism or adult intestinal toxemia botulism is due to ingestion of spores that are able to germinate and produce toxins which are subsequently absorbed and result in flaccid paralysis. This is possible if the normal intestinal flora is altered because of surgery, inflammation or use of antimicrobials (SOBEL, 2005).

d) Wound botulism

Wound botulism is caused by contamination of a wound with *C. botulinum* spores which subsequently germinate and produce BoNTs in the anaerobic milieu of the wound. Since the 1990s this form of

botulism has dramatically increased, almost exclusively among injection drug users, especially in the western U.S. (WERNER et al., 2000).

e) Unclassified cases

This category includes cases of unknown origin (DODDS & AUSTIN, 2001).

3.2.2. *C. botulinum* intoxication in animals

Naturally occurring outbreaks of *C. botulinum* have been described in several animal species, including domestic and wildlife animals (TRAMPEL et al., 2005; WOO et al., 2010; PAYNE et al., 2011). In general neurotoxin types C and D are associated with clinical outbreaks in animals; however, outbreaks in different species can be related to other types, as well.

In poultry BoNT type C is the predominantly found type (SMART & ROBERTS, 1977; TRAMPEL et al., 2005) in contrast to horses where 85% of all outbreaks were due to type B and to a far lesser extent to type A and C (WHITLOCK & MCADAMS, 2006). A toxicoinfectious form of botulism exists in foals, named the shaker foal syndrome, which is characterized by progressive muscular weakness. It results from ingested spores, germinating and producing toxin in the intestinal tract (WILKINS & PALMER, 2003). Furthermore, equine grass sickness has been considered to be a form of botulism resulting from overgrowth of *C. botulinum* type C in the intestinal tract (MCCARTHY et al., 2004).

Large outbreaks of botulism in cattle have been reported from the UK, Switzerland and Israel (LINDSTROM et al., 2010) which were mainly linked to BoNT types C and D, although types A and B were reported, too (NOTERMANS et al., 1981; SCHOCKEN-ITURRINO et al., 1990). The main risk factors for cattle ingesting BoNT is silage which supports growth of *C. botulinum* due to its anaerobic atmosphere, especially when accidentally contaminated with a dead animal (STEINMAN et al., 2006; MYLLYKOSKI et al., 2009). Furthermore, poultry litter is linked to botulism outbreaks in cattle

(PAYNE et al., 2011) as well as brewers grain (NOTERMANS et al., 1981) and bakery waste (HEIDER et al., 2001).

Recent reports suggest a new form of botulism in cattle, namely intestinal botulism or “visceral botulism”. However, its etiology and pathogenesis is not clear. Scarce studies discuss ingestion of spores that shall colonize the lower intestinal tract and may produce BoNT which is subsequently absorbed by the mucous membrane where motor nerve synapses and autonomic parasympathetic synapses are affected (BOEHNEL et al., 2001; KRUEGER et al., 2012). However to date, there is no scientific evidence supporting this hypothesis (BFR, 2010).

3.2.3. *C. botulinum* in food

C. botulinum has been detected in several foods, including food products from non-animal and animal origin, which all caused foodborne botulism outbreaks: Food products from non-animal origin include preserved olives in Finland (JALAVA et al., 2011), garlic preserved in oil in Denmark (LOHSE et al., 2003), home preserved asparagus in Italy (ZANON et al., 2006) and several home-canned vegetables in the U.S. (DATE et al., 2011). Food products from animal origin, which caused foodborne botulism, include meat from different animal species and fish, particularly raw, smoked/salted or vacuum packed products of it (RKI, 2003, 2004; KING et al., 2009; RKI, 2010).

Moreover, honey can contain spores of *C. botulinum* and has been described to be one of the risk factors for infants acquiring infant botulism (SPIKA et al., 1989). Therefore, feeding honey to infants less than one year of age is not recommended (CDC, 1998).

3.3. Public health consequences

In 2010 seven *C. botulinum* outbreaks were reported by the EFSA in Europe (EFSA & ECDC, 2012) indicating that this is a rare, but severe disease. A total of 21 cases and one death were associated with these outbreaks and they were all foodborne. The most common food source was meat (uncategorized), pork meat followed by fish and fish products, vegetables, juice and juice products.

Most often outbreaks from commercially prepared food occur due to a failure in recipes, process, packaging or lack of temperature control. However, a high percentage of botulism outbreaks were caused by homemade foods, particularly home-canned food (EFSA, 2005), which were either improper stored, handled or heated.

Furthermore, *C. botulinum* has been detected in healthy animals (DAHLENBORG et al., 2001, 2003) suggesting a possible risk of contaminating their products, namely meat via carcass contamination during the slaughter process, and milk. However, studies show that standard pasteurization conditions inactivate > 99% of BoNTs in milk (WEINGART et al., 2010) and heating food at 80℃ inactivates BoNTs as well (CDC, 1998). Therefore, proper handling and heating of food is essential to reduce the risk of ingesting BoNT via food.

4. *Yersinia enterocolitica*

4.1. *Yersinia enterocolitica* – general information

Yersinia (Y.) enterocolitica is a gram-negative, rod-shaped, motile, facultative anaerobe which is oxidase-negative, catalase-positive and ferments glucose. The genus *Yersinia* is a member of the family *Enterobacteriaceae. Y. enterocolitica* is divided into subgroups by bio- and serotyping according to the biochemical activity and lipopolysaccharide (LPS) O antigens. Most human

and animal infections are due to biotype 1B, 2, 3, 4, and 5, whereas 1A is often referred to as an environmental biovar. Common serovars obtained from humans are O:3, 9, 5, and 27. In animals, primarily cattle, serotype O:9 is of particular importance as LPS from *Y. enterocolitica* cross-reacts with LPS from *Brucella spp.* and may induce false-positive reactions in brucella agglutination tests (WEYNANTS et al., 1996).

Virulence factors of *Y. enterocolitica* consist of adhesins which are encoded on the chromosome, as invasin and *ail* which mediates bacterial attachment and invasion and also protects *Y. enterocolitica* from nonspecific destruction by the complement system. The *ail* gene is only found in virulent serovars. Furthermore, *Y. enterocolitica* secrete heat-stable enterotoxins (Yst) which are also encoded on the chromosome. Virulent *Y. enterocolitica* carry a plasmid (pYV) which encodes proteins called YopB (*Yersinia* outer proteins), -D, -E, -H, -M, -N, -O, -P, -Q, and -R and YadA (*Yersinia* adhesin), YlpA, LcrV. These proteins are virulence factors with a number of them acting on host cells to subvert immune defense (DOYLE et al., 2001).

4.2. Epidemiology

4.2.1. *Y. enterocolitica* infection in humans

Y. enterocolitica is a worldwide foodborne pathogen causing yersiniosis which is the third most commonly reported zoonosis in Europe (EFSA & ECDC, 2012). In 2010 6,776 confirmed cases were reported in the EU with 3,346 confirmed cases reported from Germany (EFSA & ECDC, 2012). Sporadic outbreaks of yersiniosis occur most often in children less than five years of age and the highest incidence is among one year olds with 49 per 100,000 population in 2010 in Germany (RKI, 2012). Most symptomatic infections result in self-limiting diarrhea, however, suppurative and autoimmune complications can occur, as well.

In Europe serovar O:3 is the most frequently isolated one from humans, followed by serovar O:9 and the most frequent biovar worldwide is biovar 4 (Table 4).

TABLE 4. Geographic distribution of serovars and biovars of *Y. enterocolitica* (BOTTONE, 1997).

Geographic distribution	Serovar	Biovar
United States	O:8	1B
	O:3	4
Canada	O:3	4
Europe	O:3	4
	O:9	2
Japan	O:3	4
South Africa	O:3	4

4.2.2. *Y. enterocolitica* infection in animals

Natural *Y. enterocolitica* infections in animals have rarely been reported: In goats and sheep it causes enteritis and microabscesses in the intestine (SLEE & BUTTON, 1990) and sporadic ovine abortion (CORBEL et al., 1990). It was furthermore detected in chinchillas and other rodents causing enteritis (WUTHE & ALEKSIC, 1992). Experimental infection of rodents with *Y. enterocolitica* resulted in purulent meningoencephalitis, catarrhal pneumonia and arthritis (DE LOS TOYOS et al., 1990; VESSELINOVA et al., 2001). In experimentally infected pigs histological findings were meningoencephalitis as well as catarrhal enteritis and necrotic tonsillitis (NAJDENSKI et al., 1998).

Moreover, *Y. enterocolitica* could be detected in feces of clinically healthy animals, as in pigs, cattle, sheep (MCNALLY et al., 2004) and even companion animals and rodents (BUCHER et al., 2008). *Y. enterocolitica* though, is more frequently detected in healthy pigs and pigs at the slaughterhouse than in other animal species (EFSA & ECDC, 2012).

4.2.3. *Y. enterocolitica* in food

Pathogenic *Y. enterocolitica* strains were frequently found in pork meat, especially edible offal and tongue (FREDRIKSSON-AHOMAA et al., 1999; BUCHER et al., 2008; MESSELHÄUSSER et al., 2011). Moreover, other sources of foodborne transmission were described in the U.S.: A case of human yersiniosis was reported after drinking pasteurized milk (CDC, 2011) and another American study reported *Y. enterocolitica* in bulk tank milk (JAYARAO et al., 2006).
Furthermore, *Y. enterocolitica* was detected in beef and meat originating from chicken and turkey (MAYRHOFER et al., 2004) as well as in ready-to-eat salad (MACDONALD et al., 2012).

4.3. Public health consequences

Animals, particularly pigs and their products, are considered to be the main reservoir for human pathogenic *Y. enterocolitica* as they mainly harbor biotype 4 (O:3). Human pathogenic biotype 4 (O:3) and 2 (O:9) have been detected in other animals as well, namely cattle, sheep, dogs and cats however, with a far lesser extent (EFSA & ECDC, 2012). Nevertheless, they still can act as a reservoir. The main risk factor for acquiring *Y. enterocolitica* appears to be eating raw or undercooked food, especially pork which is a preventable cause of foodborne disease due to proper preparing and handling of food (RKI, 2012).

III. PUBLICATIONS

3.1. Publication 1

Prevalence of extended-spectrum β-lactamase-producing *Escherichia coli* on Bavarian dairy and beef cattle farms

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Prevalence of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* on Bavarian Dairy and Beef Cattle Farms

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Extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* strains are believed to be widely distributed among humans and animals; however, to date, there are only few studies that support this assumption on a regional or countrywide scale. Therefore, a study was designed to assess the prevalence of ESBL-producing *E. coli* in dairy cows and beef cattle in the southern part of Bavaria, Germany. The study population included 30 mixed dairy and beef cattle farms and 15 beef cattle farms. Fecal samples, boot swabs, and dust samples were analyzed for ESBL-producing *E. coli* using selective media. PCR was performed to screen for CTX-M and *ampC* resistance genes. A total of 598 samples yielded 196 (32.8%) that contained ESBL-producing *E. coli*, originating from 39 (86.7%) of 45 farms. Samples obtained from mixed farms were significantly more likely to be ESBL-producing *E. coli* positive than samples from beef cattle farms (fecal samples, $P < 0.001$; boot swabs, $P = 0.014$; and dust samples, $P = 0.041$). A total of 183 isolates (93.4%) of 196 ESBL-producing *E. coli*-positive strains harbored CTX-M genes, CTX-M group 1 being the most frequently found group. Forty-six additional isolates contained *ampC* genes, and 5 of the 46 isolates expressed a bla_{CMY-2} gene. The study shows that ESBL-producing *E. coli* strains are commonly found on Bavarian dairy and beef cattle farms. Moreover, to our knowledge, this is the first report of the occurrence of bla_{CMY-2} in cattle in Germany.

An increasing number of extended-spectrum β-lactamases (ESBLs) have been identified in *Enterobacteriaceae* during the last few years. They were not only detected in humans (1, 2), but also in a broad range of animal species ranging from companion animals (3–5) to food animals (6–10), and they could also be found in food (11, 12). Extended-spectrum β-lactamases are plasmid-encoded enzymes that inactivate a large number of β-lactam antibiotics, including extended-spectrum and very-broad-spectrum cephalosporins and monobactams. They are also commonly inhibited by β-lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam. Furthermore, resistance to broad-spectrum cephalosporins can be due to overexpression of chromosomal or plasmid-mediated AmpC enzymes, encoded by genes such as bla_{CMY-2}. These enzymes also confer resistance to cephamycins and cannot be inhibited by β-lactamase inhibitors. Since first emerging in the late 1980s, bla_{CTX-M} genes have become the most common genes encoding ESBL in many countries. They have been reported to be isolated from different food-producing animals that were recognized as reservoirs for ESBL-producing *E. coli* (13). Several studies indicate that these resistance genes are disseminated through the food chain or via direct contact with humans and animals (14, 15). The data about ESBL-producing bacteria in food animals in Germany are very limited. To date, only one publication on the detection of ESBL-producing *E. coli* in food animals in Germany has been available (16). Therefore, this study was conducted to estimate the prevalence of ESBL-producing *E. coli* in healthy cattle, evaluating different ages and farm types.

MATERIALS AND METHODS

Study population and sampling. In this study, 45 randomly selected farms were enrolled in the southern part of Bavaria, Germany, from summer 2011 until summer 2012. Thirty farms were mixed dairy and beef cattle farms where three groups (calves, lactating dairy cows, and beef cattle) were sampled and tested. The mean herd sizes were 57 ± 37.1 cows, 24 ± 16.2 calves, and 44 ± 35.5 beef cattle. The cows enrolled in this study were between 2 and 15 years old, and the calves were at least 2 days old, with the oldest being 6 months of age. Half of the farms housed dairy cows in free-stall barns and half of them in tie-stall barns. Most farms used hutches and individual pens for their calves. All beef cattle were housed in pens with six to eight animals each. The mean ages at slaughter were 4 ± 0 months for veal calves and 20 ± 2.2 months for beef cattle. Fifteen farms were exclusively beef cattle farms, where the youngest and the oldest groups were tested. On most farms, beef cattle were housed in pens with six to eight animals each. The mean size of herds was 130 ± 108.5, and the mean age of bulls at slaughter was 20 ± 2.3 months. In addition to the 45 survey farms, 9 combined cow and calf herds (the "cow-calf" group) and one beef cattle farm were enrolled as a control group not having used antimicrobials for at least half a year.

From every group on each farm, three pooled fecal samples, one dust sample, and a pair of boot swabs from the feed alley were collected (Table 1). On mixed farms, 10 fecal samples from cows were collected for one pooled fecal sample and six fecal samples from calves and six fecal samples from bulls were included into one pooled fecal sample from each group. On beef cattle farms, one pooled fecal sample represented six to eight bulls from one pen. On one farm, only two pooled fecal samples could be collected from calves and beef cattle as there were only two calves in each group. If dust could not be collected, it was replaced by another pair of boot swab samples.

Cow and calf herds were tested as one cow-calf group. Mostly these animals were on pasture, so there was only the possibility of taking boot swabs. Where possible, a dust sample was taken also (Table 1).

Microbiological methods and susceptibility tests. ESBL-producing *E. coli* strains were detected using an enrichment procedure: A 5-g aliquot

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TABLE 1 Numbers and distributions of samples collected from farms

Sample source (*n*)	No. of samples		
	Fecal (3/group)	Boot swabs (1 pair/group)	Dust (1/group)
Mixed farms (30)			
Cows	90	31	29[b]
Calves	89[a]	30	30
Beef cattle	89[a]	31	29[b]
Sum	268	92	88
Beef cattle farms (15)			
Youngest group	45	15	15
Oldest group	45	16	14[b]
Sum	90	31	29
Control group (10)			
Cow-calf herds (9)	27	9	3[c]
Beef cattle farms (1)	6	2	2
Sum	33	11	5

[a] There were not enough animals for a third fecal sample.
[b] A dust sample was replaced with another pair of boot swabs.
[c] If not on pasture, a dust sample was collected as well.

of the fecal sample was placed in 45 ml Luria-Bertani (LB) broth (Sigma-Aldrich, Germany), 1 ml of the dust suspension (0.1 g of the dust sample in 10 ml phosphate-buffered saline [PBS] [Merck, Germany] with 0.01% Tween20 [Merck, Germany]) in 9 ml of LB broth, and a pair of boot swabs in 225 ml of LB broth. The LB broth was incubated for 24 h under aerobic conditions at ±37°C, and 10 μl of each sample was streaked onto MacConkey agar (MCA) (Oxoid, United Kingdom) containing cefotaxime (1 μg/ml) (Sigma-Aldrich, Germany), which was incubated for another 24 h under aerobic conditions at ±37°C.

Fecal samples and dust suspensions from 35 farms (21 mixed farms and 14 beef cattle farms) were additionally plated directly onto MCA with cefotaxime, omitting the enrichment step.

Coliform colonies growing on MCA with cefotaxime were subcultured on blood agar (Oxoid, United Kingdom), biochemically identified as *E. coli*, and phenotypically identified as ESBL or AmpC producers using the Phoenix system with panel NMIC/ID-76 (Becton, Dickinson, Germany). The breakpoints used were those defined by EUCAST for *Enterobacteriaceae* (http://www.eucast.org).

Molecular characterization. Isolates that were phenotypically identified as ESBL producers were screened for the presence of $bla_{\text{CTX-M}}$ genes by real-time PCR using detection systems described by Birkett et al. (17). DNA was purified with the QIAamp DNA stool minikit (Qiagen, Germany). The presence of *ampC* β-lactamase genes was detected in phenotypic AmpC isolates by PCR too (18). Phenotypically ESBL-positive but genotypically negative CTX-M and AmpC isolates were also screened for bla_{TEM} and bla_{SHV} by real-time PCR (19), and DNA was sequenced afterwards.

Survey data. On the day of the sample collection, the farmers answered a survey assessing basic herd information: e.g., demographic information, animal purchase, housing, performance, hygiene management, and use of antimicrobials. Information was collected for all animals of the tested groups and analyzed to assess possible risk factors for the spread of ESBL-producing *E. coli*.

Statistical analysis. The data were entered into MS Excel (version 2010; Microsoft) and analyzed using Epi Info (StatCalc, version 6; CDC, Atlanta, GA) and SPSS (version 19; IBM, Germany). For categorical data, chi-square tests were performed. If the sample size was less than 30, a Yates' correction was applied. If any of the expected cell frequencies was less than 5, Fisher's exact test was used. Continuous data were examined visually for normal distribution (box plot) and analyzed for differences between groups using Mann-Whitney U test and Kruskal-Wallis test. Differences with $P \leq 0.05$ were considered significant.

RESULTS

Between July 2011 and June 2012, 45 farms were screened for ESBL-producing *E. coli*. In total, 598 samples were collected and in 196 (32.8%) samples, ESBL-producing *E. coli* strains were detected. At the farm level, at least one sample of 39 (86.7%) farms was found to be positive for ESBL-producing *E. coli*: i.e., 28 of 30 (93.3%) mixed farms and 11 of 15 (73.3%) beef cattle farms tested positive. The prevalence of ESBL-producing *E. coli* on mixed farms was 38% for all samples, compared to 17.3% of all samples from beef cattle farms and 6.1% of samples from the control group. The highest rates of ESBL-producing *E. coli* on mixed farms could be detected in calves, with 56.2% of fecal samples harboring ESBL-producing *E. coli*, followed by cows (41.1% of fecal samples) and beef cattle (21.4% of fecal samples) (Table 2).

In the control group, 3 of 10 (30%) farms were positive for ESBL-producing *E. coli*, with one positive boot swab sample on

TABLE 2 Percentages of ESBL-producing *E. coli*-positive samples and confidence intervals

Sample source	No. of samples/total (%) [CI]		
	Fecal	Boot swabs	Dust
Mixed farms			
Cows	37/90 (41.1) [31.5–51.4]	14/31 (45.2) [29.2–62.2]	5/29 (17.2) [7.6–34.6]
Calves	50/89 (56.2) [45.8–66.0]	21/30 (70) [52.1–83.3]	12/30 (40) [24.6–57.7]
Beef cattle	19/89 (21.4) [14.1–31.0]	12/31 (38.7) [23.7–56.2]	0/29 (0) [0.0–11.7]
Sum	106/268 (39.6) [33.9–45.5]	47/92 (51.1) [41.0–61.1]	17/88 (19.3) [12.4–28.8]
Beef cattle farms			
Youngest group	12/45 (26.7) [16.0–41.0]	3/15 (20) [7.1–45.2]	1/15 (6.7) [1.2–29.8]
Oldest group	5/45 (11.1) [4.8–23.5]	5/16 (31.3) [14.2–55.6]	0/14 (0) [0.0–21.5]
Sum	17/90 (18.9) [12.1–28.2]	8/31 (25.8) [13.7–43.3]	1/29 (3.5) [0.6–17.2]
Total sum for mixed farms and beef cattle farms	123/358 (34.4) [29.6–39.4]	55/123 (44.7) [36.2–53.5]	18/117 (15.4) [10.0–23.0]
Control group	0/33 (0) [0.0–10.4]	3/11 (27.3) [9.8–56.6]	0/5 (0) [0.0–43.5]

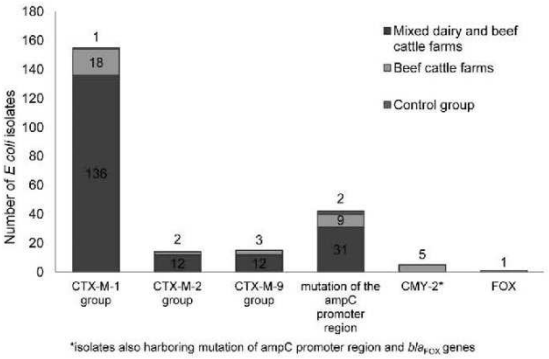

FIG 1 Distribution of β-lactamase genes in 184 ESBL-producing *E. coli* isolates and 48 AmpC-producing *E. coli* isolates from mixed dairy and beef cattle farms and beef cattle farms.

each farm (Table 2). No ESBL-producing *E. coli* strains could be detected in fecal or dust samples from control farms.

An overall significant difference in prevalence was shown between mixed farms and beef cattle farms in comparison to the control group. Mixed and beef cattle farms were more likely to harbor samples containing ESBL-producing *E. coli* strains than the control group ($P < 0.001$, relative risk [RR] = 5.35; confidence interval [CI], 1.8 to 16.1).

Samples obtained from mixed farms were significantly more often positive for ESBL-producing *E. coli* than samples from beef cattle farms (fecal samples, $P < 0.001$, RR = 2.09, CI, 1.3 to 3.3; boot swabs, $P = 0.014$, RR = 1.98, CI, 1.1 to 3.7; and dust samples, $P = 0.041$, RR = 5.60; CI, 0.8 to 40.3). On mixed farms, the overall recovery of ESBL-producing *E. coli* was significantly more likely for calves (55.7%) than for cows (37.3%) ($P = 0.002$; RR, 1.49; CI, 1.2 to 1.9). Fecal samples and boot swabs from calves were significantly more often positive than those from dairy cows (fecal samples, $P = 0.044$, RR = 1.37, CI, 1.0 to 1.9; boot swabs, $P = 0.049$, RR = 1.55, CI, 1.0 to 2.4). On mixed farms, the prevalence of ESBL-producing *E. coli* for all samples differed significantly between calves and beef cattle (fecal samples, $P < 0.001$, RR = 2.63, CI, 1.7 to 4.1; boot swabs, $P = 0.014$, RR = 1.81, CI, 1.1 to 3.0; dust samples, $P < 0.001$).

Farms of the control group showed no positive fecal samples ($P < 0.001$ compared to mixed farms, $P = 0.006$ compared to beef cattle farms).

Among all farms, 35 were screened for ESBL-producing *E. coli* with and without the enrichment step. In 41 of 273 (15%) fecal samples, ESBL-producing *E. coli* strains could be detected directly and with enrichment, compared to 79 (28.9%) of only enriched fecal samples. All 13 dust samples needed an enrichment step to detect ESBL-producing *E. coli*.

PCR was performed on all 196 ESBL-producing *E. coli* isolates to screen for CTX-M genes. A total of 183 isolates (93.4%) harbored CTX-M genes, CTX-M group 1 being the most frequently found group (Fig. 1). One isolate was identified as a TEM-52-harboring *E. coli* strain. Phenotypically ESBL-positive but genotypically negative CTX-M isolates were categorized as ESBL producers as the performed PCRs screened for the most common resistance genes but didn't include all existing resistance genes.

In addition, 48 separate isolates that were phenotypically identified as AmpC producers using the Phoenix system (Becton Dickinson, Germany) were screened for genes encoding AmpC β-lactamases, which were found in 46 of the 48 isolates. Most isolates harbored mutations in the AmpC promoter region at base −42, a C→T transition, and at position −32, a T→A transversion. This mutation leads to overexpression of the chromosomal AmpC β-lactamase genes and overproduction of these enzymes (20). On mixed farms, AmpC production was only related to mutations in the AmpC promoter region, in contrast to beef cattle farms, where 6 isolates showed a plasmid-encoded AmpC production (Fig. 1). Twelve isolates harbored gene sequences for both CTX-M and AmpC. Phenotypically AmpC-positive but genotypically negative isolates were categorized as AmpC producers as the performed PCR did not screen for all existing resistance genes.

All three phenotypic ESBL-producing *E. coli* strains isolated from the control group were screened for CTX-M genes. Two phenotypic AmpC isolates were screened for genes encoding AmpC β-lactamases. One isolate (33.3%) carried a resistance gene belonging to CTX-M group 1, and both isolates were positive for a mutation in the AmpC promoter region (Fig. 1).

MICs of 20 antimicrobial drugs were determined for all 196 ESBL-producing *E. coli* isolates (Table 3). All isolates showed susceptibility to carbapenems, except for one ertapenem-resistant isolate from a beef cattle farm. A significant difference in susceptibility of isolates associated with different farm types could be detected: ESBL-producing *E. coli* from beef cattle farms were significantly less likely to be resistant against aztreonam ($P = 0.030$; RR = 1.29; CI, 1.0 to 1.7), gentamicin ($P = 0.0014$; RR = 3.33; CI, 1.3 to 8.3), tobramycin ($P = 0.008$; RR = 2.12; CI, 1.1 to 4.1), ciprofloxacin ($P < 0.001$; RR = 3.82; CI, 1.5 to 9.5), and trimethoprim-sulfamethoxazole ($P = 0.017$; RR = 1.55; CI, 1.0 to 2.4) than isolates from mixed farms. Resistance to fosfomycin, though, was found more often on beef cattle farms ($P = 0.008$; RR = 19.62; CI, 2.1 to 181.6) than mixed farms.

Of the 196 isolates, 30 (15.3%) were resistant to two substance classes (Fig. 2). If an isolate was classified as intermediate for a group, it was categorized as resistant in this type of analysis. Altogether, 39 isolates (19.9%) showed resistance against three substance classes. Most of the isolates (127 [64.8%]) were multiresistant (resistant to three or more substance classes).

Survey. The survey showed that 30 of 30 (100%) mixed farms and 14 of 15 (93.3%) beef cattle farms used antimicrobials for the last 6 months; 96.7% of mixed farms and 71.4% of beef cattle farms administered β-lactam antimicrobials. Furthermore, the data showed that ESBL-producing *E. coli* strains were significantly more often detected in fecal samples from calf groups treated with antimicrobials ($P = 0.041$) than in untreated groups: ESBL-producing *E. coli*-positive fecal samples were detected on 24 of 30 farms, with 22 farms reporting common use of antimicrobials, no farms reporting sporadic use, and 2 farms reporting no use of antimicrobials in the calf group. ESBL-producing *E. coli*-negative fecal samples from the calf group were found on 6 of the 30 farms, where 3 farms reported common use of antimicrobials, 1 farm reported sporadic use, and 2 farms reported no use of antimicrobials in this group.

A relationship between feeding waste milk (which may contain remnants of antibiotics) to calves and a higher detection rate of ESBL-producing *E. coli* in fecal samples from this group was sta-

TABLE 3 Resistance patterns of bovine ESBL-producing *E. coli* isolates

Antimicrobial(s)	No. (%) of isolates from:					
	Mixed dairy and beef cattle farms (n = 170)			Beef cattle farms (n = 26)		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Ampicillin	0	0	170 (100)	0	0	26 (100)
Cefazolin	0	0	170 (100)	0	0	26 (100)
Cefuroxime	0	0	170 (100)	0	0	26 (100)
Cephalexin	1 (0.6)	0	169 (99.4)	1 (3.9)	0	25 (96.2)
Cefotaxime	1 (0.6)	7 (4.1)	162 (95.3)	0	4 (15.4)	22 (84.6)
Ceftazidime	33 (19.4)	82 (48.2)	55 (32.4)	10 (38.5)	10 (38.5)	6 (23.1)
Imipenem	170 (100)	0	0	26 (100)	0	0
Meropenem	170 (100)	0	0	26 (100)	0	0
Ertapenem	170 (100)	0	0	25 (96.2)	0	1 (3.9)
Aztreonam	1 (0.6)	26 (15.3)	143 (84.1)	1 (3.9)	8 (30.8)	17 (65.4)
Nitrofurantoin	164 (96.5)	0 (0)	6 (3.5)	23 (88.5)	0	3 (11.5)
Gentamicin	78 (45.9)	5 (2.9)	87 (51.2)	22 (84.6)	0	4 (15.4)
Tobramycin	73 (42.9)	0	97 (57.1)	19 (73.1)	0	7 (26.9)
Amikacin	169 (99.4)	1 (0.6)	0	26 (100)	0	0
Ciprofloxacin	68 (40.0)	2 (1.2)	100 (58.8)	22 (84.6)	0	4 (15.4)
Trimethoprim-sulfomethoxazole	48 (28.2)	0	122 (71.8)	14 (53.8)	0	12 (46.2)
Piperacillin-tazobactam	141 (82.9)	5 (2.9)	24 (14.1)	22 (84.6)	0	4 (15.4)
Amoxicillin-clavulanic acid	80 (47.1)	0	90 (52.9)	12 (46.2)	0	14 (53.8)
Colistin	170 (100)	0	0	26 (100)	0	0
Fosfomycin	169 (99.4)	0	1 (0.6)	23 (88.5)	0	3 (11.5)

tistically not significant (P = 0.055; median for calves without waste milk, 0.33; median for calves with waste milk, 0.83).

On mixed farms, an association could be demonstrated between the number of animals on a farm and ESBL-producing *E. coli*-positive samples from cows: positive samples could be detected more often from farms with a higher number of animals on the farm (P = 0.025; median number of animals on farms where the cow group is positive for ESBL-producing *E. coli*, 155; median number of animals on farms where the cow group is negative for ESBL-producing *E. coli*, 98) and a higher number of animals in the cow group (P = 0.019; median number of cows on farms where the cow group is positive for ESBL-producing *E. coli*, 60; median number of cows on farms where the cow group is negative for ESBL-producing *E. coli*, 40). Furthermore, the data showed that positive fecal samples from beef cattle were more likely to be found on farms purchasing greater numbers of animals for beef cattle (P = 0.045; median number of purchased beef cattle from farms where fecal samples obtained from the beef cattle group were positive for ESBL-producing *E. coli*, 20; median number of purchased beef cattle from farms where no fecal samples from the beef cattle group were positive for ESBL-producing *E. coli*, 10).

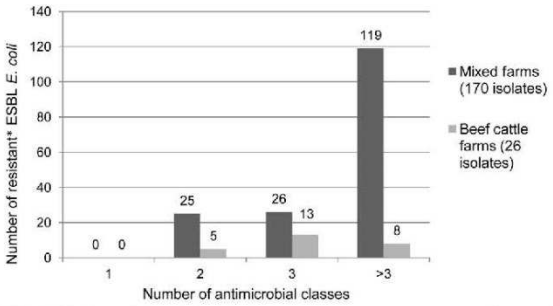

FIG 2 ESBL-producing *E. coli* isolates resistant to one or more classes of antimicrobial substances. The asterisk indicates that if an isolate was classified as intermediate for a group, it was categorized as resistant.

On beef cattle farms the survey showed similar findings: on farms purchasing higher numbers of animals, ESBL-producing *E. coli* was more likely to be detected (P = 0.022; median number of purchased animals on farms with ESBL-producing *E. coli*, 130; median number of animals on farms without ESBL-producing *E. coli*, 19) in at least one sample.

DISCUSSION

This study was designed to assess the status of ESBL-producing *E. coli* on cattle farms typical for Bavaria and to elucidate possible risk factors for their spread. Focus was laid on herd and group status, and therefore, individual animal aspects were not evaluated. The ESBL-producing *E. coli* status was defined at the group or herd level, where this technique was suggested in cattle (21). The results show that ESBL-producing *E. coli* strains were much more frequently detected than expected on Bavarian mixed and beef cattle farms. They could be detected on 86.7% of all farms, mixed farms being positive more often than beef cattle farms. The prevalences of ESBL-producing *E. coli*-positive fecal samples on mixed farms were 39.6% and 18.9% on beef cattle farms. This seems to be an increase, as such strains have only sporadically been described in Germany before (16). However, this could also be due to differences in the detection methods as in this study ESBL-producing *E. coli* strains were selected using enrichment and selective media (MCA containing cefotaxime). Guerra et al. (16), however, did not test samples from field trials, but received *E. coli* isolates from different labs wanting them to be typed. These isolates have not been selected with special media, which has an important effect on the results. Other European studies reported common occurrence of ESBL-producing *E. coli* in cattle in Swit-

zerland (22), France (23), Denmark (24), and the United Kingdom (8).

This high prevalence of ESBL-producing *E. coli* could be based on using enrichment procedures for selection. With 92 enriched fecal and dust samples being positive for ESBL-producing *E. coli* compared to 41 positive samples (44.6%) without enrichment, ESBL-producing *E. coli* strains could be isolated more than twice as often using enrichment procedures. Even small numbers of *E. coli* strains were more likely to be found using the enrichment media. Therefore, we suggest using an enrichment procedure for the screening of ESBL that is especially recommended for dust samples.

An overall significant difference in prevalences exists between mixed and beef cattle farms using antimicrobials and the control group not having used antimicrobials for at least half a year ($P < 0.001$; RR = 5.35; CI, 1.8 to 16.1), although the control group consisted of another farm type for technical reasons. Different studies described that the use of antimicrobials exerts a dominant selective pressure and may favor the spread of resistance genes (25–27). In this study, 100% (30/30) of mixed farms and 93.3% (14/15) of beef cattle farms used antimicrobials for the last 6 months. β-Lactam antimicrobials have been used on 96.7% of mixed farms and on 71.4% of beef cattle farms. We found ESBL-producing *E. coli* even on farms that did not use antimicrobials of this group, but the use of non-β-lactam antimicrobials can select for ESBL resistance genes as well, since the resistance determinants against cephalosporins, aminoglycosides, tetracycline, and sulfonamides are often situated on the same plasmid (28). The use of any of these antimicrobials can coselect for all other ones. Plasmids that carry antimicrobial resistance genes can also carry genes mediating resistance against disinfectants, heavy metal tolerance, virulence, and metabolic functions and, therefore, could be coselected (29).

The overall prevalence of ESBL-producing *E. coli* was much higher in calves (55.7%) than in cows (37.3%) ($P = 0.002$; RR = 1.49; CI, 1.2 to 1.9). This is consistent with recently published studies in Great Britain (30, 31). ESBL-producing *E. coli*-positive fecal samples were more often recovered from calves on farms using antimicrobials ($P = 0.041$). Using antimicrobials can potentially contribute to the maintenance and spread of ESBL. Berge et al. (32), though, observed highly resistant *E. coli* in dairy heifers not exposed to antimicrobials. They concluded that an individual antibiotic therapy could lead to selective pressure enough to establish a resistant gene pool in the farm-level bacterial population.

The survey data showed that calves being fed waste milk ($P = 0.055$; median, 0.33; SE, 0.83) harbor more ESBL-producing *E. coli*-positive fecal samples, however, not significantly. The observation of the effect of feeding waste milk (which may contain antibiotics at low concentrations) to calves suggests that this practice may act as a selection pressure and may be an advantage for bacteria with resistance to antimicrobials. Consistent proposals have been stated in a previous study (32) where Berge et al. hypothesized that feeding waste milk may play a role in selecting resistant bacterial populations on farms.

We screened all 196 phenotypic ESBL-producing *E. coli* isolates for the bla_{CTX-M} genes and found that 93.4% of isolates were positive. This indicates that bla_{CTX-M} genes are present on most Bavarian mixed and beef cattle farms. The genes are frequently responsible for resistance to extended-spectrum and very-broad-spectrum cephalosporins in *Enterobacteriaceae* in Europe, group 1 (CTX-M-1 and -15) being the predominant *bla* genes in western and northern European countries (33). CTX-M-9 group has been isolated from animals in Spain (34), France (35), and the United Kingdom (36), whereas CTX-M group 2 has been mainly described in South America and Japan (37). These findings are consistent with the observation in this study as $bla_{CTX-M-1}$ group genes were predominantly detected, as were, sporadically, bla_{CTX-M} genes belonging to groups 2 and 9.

In addition, 48 phenotypic AmpC *E. coli* isolates were screened for the presence of mutations of the promoter region and plasmid-mediated AmpC. A predominance of mutation of the promoter region was found; however, five isolates with bla_{CMY-2} were yielded from three beef cattle farms. To our knowledge, this is the first time that bla_{CMY-2} has been detected in cattle in Germany. Before, bla_{CMY-2} was described in *Salmonella* spp. from poultry only (38). However, studies from different European countries show that bla_{CMY-2} genes are well described in cattle (23, 39–41).

Conclusion. We observed that ESBL-producing *E. coli* strains are commonly found on Bavarian dairy and beef cattle farms, recognizing that our study provides data only for a circumscribed area in Germany. However, the results were consistent with other European studies. There is a paucity of studies that show a countrywide prevalence in Germany and other European countries. Additional nationwide surveys would be necessary to identify the possible spread of ESBL-producing *E. coli*, its transmission dynamics, and risk factors. Different countries, including Germany, have already started screening for ESBL-producing *E. coli* on a wider scale, but final results are not yet available.

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3.2. Publication 2

Occurrence of zoonotic *Clostridia* and *Yersinia* in healthy cattle

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Occurrence of Zoonotic *Clostridia* and *Yersinia* in Healthy Cattle

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ABSTRACT

Zoonotic pathogens are a frequent cause of disease worldwide. This study was designed to determine the occurrence of *Clostridium difficile*, *Clostridium botulinum*, and *Yersinia enterocolitica* in cattle in southern Bavaria, Germany. The study population included 49 farms; 34 were dairy farms (30 also fattening beef cattle) and 15 were solely beef cattle farms. Fecal and dust samples were collected from summer 2011 to summer 2012 and analyzed using a combination of enrichment procedures and real-time PCR. For the detection of *C. difficile*, samples were screened for the presence of the *tpi* gene and toxin genes *tcdA*, *tcdB*, and *cdtA*. Samples also were screened for genes for *C. botulinum* toxins A through F and for the *ail* gene of *Y. enterocolitica*. Of 506 samples, *C. difficile* genes were found in 29 samples (5.7%): 25 samples from dairy farms and 4 samples from beef cattle farms. Toxin genes were identified in 17 samples, with toxigenic profiles of $A^+B^+CDT^-$, $A^+B^-CDT^+$, and $A^+B^+CDT^+$. *C. botulinum* toxin genes were not detected in fecal samples from cattle, but the gene for toxin B was detected in 1 (0.8%) of 125 dust samples. *Y. enterocolitica* genes were found in 6 (1.6%) of 382 fecal samples from three dairy farms and one beef cattle farm. This study revealed that *C. difficile* and *Y. enterocolitica* are rare on cattle farms in Bavaria, Germany. In contrast to results of previous studies, *C. botulinum* was not detected in fecal samples but was found very rarely in dust samples from the cattle environment.

Zoonotic pathogens are an important source of human infections. In 2010, 5,262 foodborne outbreaks were reported by 27 European Union member states and comprised 43,473 human cases *(16)*. Pathogenic bacteria may be present in farm animals and in products derived from food animals, which could act as vectors for transmitting pathogenic bacteria to humans. Among other well-known zoonotic pathogens, *Clostridium difficile*, *Clostridium botulinum*, and *Yersinia enterocolitica* play a significant role in human medicine. *C. difficile* causes serious nosocomial diarrhea in humans, mostly associated with prior use of antibiotics. An increase in incidence and severity of this infection has been reported in hospitals; the diagnosis in the United States doubled from 31 per 100,000 people in 1996 to 61 per 100,000 people in 2003 *(38)*. This increase in *C. difficile* infections in hospitals seems to be due in part to new hypervirulent strains, e.g., PCR ribotypes 027 *(35, 37)* and 078 *(4, 24)*. Type 078 was reported as the predominant PCR ribotype in pigs and calves *(30)*, and in a Dutch study a high level of genetic relatedness was found between human and porcine strains *(24)*, suggesting transmission between animals and humans. *C. difficile* also has been detected in various farm animals, pets, and wildlife *(2, 5, 6, 46, 53, 58)*, although to date no route of transmission between animals and humans has been identified. The emergence of community-acquired *C. difficile* infection in populations previously thought to be at low risk, such as young adults and children without exposure to antimicrobials or hospitals, has been recently described *(33)*.

Seven outbreaks of *C. botulinum* infection in Europe were reported by the European Food Safety Authority in 2010, with a total of 21 cases and one death. All outbreaks were foodborne *(16)*, demonstrating that acute foodborne botulism is a rare but severe disease. This disease is often associated with contaminated meat, fish, or vegetables *(16)* and has been reported in cattle and dairy products *(1, 36, 44)*. In 1996, a botulism outbreak was associated with mascarpone cream cheese in Italy *(1)*. *C. botulinum* is commonly present in the environment *(26, 52)* and may be transmitted to food-producing animals, which are thus a potential source for *C. botulinum* infection.

Y. enterocolitica is a worldwide zoonotic pathogen; yersiniosis is the third most commonly reported zoonosis in Europe *(16)*. In 2010, 6,776 confirmed cases were reported in the European Union, and 3,346 of these confirmed cases occurred in Germany *(16)*. Pigs and pig meat were described as a major source of foodborne yersiniosis *(16, 19–23)*; raw milk also was associated with human yersiniosis *(10)*. *Y. enterocolitica* has been isolated from various farm animals such as pigs, cattle, and goats; however, isolation rates from pigs are higher than those from cattle and goats. Cats and dogs also appear to be reservoirs for *Y. enterocolitica (17, 18, 51)*.

The importance of *C. difficile*, *C. botulinum*, and *Y. enterocolitica* as zoonotic pathogens in human medicine is

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well documented. However, only limited data are available concerning their prevalence in animals, especially cattle, which could act as potential reservoirs. In the present study, the occurrence of *C. difficile*, *C. botulinum*, and *Y. enterocolitica* was assessed in healthy dairy and beef cattle of various ages.

MATERIALS AND METHODS

Study population and sampling. Forty-nine randomly selected farms from the southern part of Bavaria, Germany, participated in this study from summer 2011 to summer 2012 to determine the occurrence of *C. difficile*, *C. botulinum*, and *Y. enterocolitica*. The 49 farms included 34 dairy farms, 30 of which were also fattening beef cattle, and 15 beef cattle farms. On dairy farms, calves, lactating cows, and beef cattle were tested. Mean (± standard deviation) herd size was 59 ± 39.4 cows, 25 ± 16.5 calves, and 44 ± 35.5 beef cattle. Dairy cows were housed in free-stall barns on 18 farms and in tie-stall barns on 16 farms. Most farms used hutches or individual pens for calves, and all farms housed beef cattle in pens with six to eight animals each. The mean age at slaughter was 4 ± 0 months for veal calves and 20 ± 2.2 months for beef cattle.

On the 15 beef cattle farms, the youngest and the oldest animal groups were tested. Bulls were mainly housed in pens with six to eight animals each. The mean size of herds was 130 ± 108.5 animals, and the mean age of bulls at slaughter was 20 ± 2.3 months.

On each farm, three pooled fecal samples and one dust sample were collected from each animal group. On dairy farms, one pooled fecal sample represented 10 fecal samples from cows, 6 fecal samples from calves, and 6 fecal samples from bulls. On beef cattle farms, six to eight fecal samples from one pen were included in one pooled fecal sample.

Laboratory testing: *C. difficile*. Ten grams of each pooled fecal sample and 0.1 g of each dust sample were placed into 90 ml and 10 ml, respectively, of Trypticase-peptone-glucose-yeast (TPGY) broth (Merck, Darmstadt, Germany) supplemented with 0.1% sodium taurocholate (Carl Roth, Karlsruhe, Germany) and *C. difficile* selective supplement (CDMN selective supplement, Oxoid, Basingstoke, UK), respectively, and heated for 10 min at 60°C. The enrichment broth was incubated anaerobically for 72 h at 37°C in anaerobic jars (BD, Franklin Lakes, NJ) with AnaeroGen sachets (Oxoid) to create an anaerobic atmosphere.

DNA from 1 ml of the broth was purified with the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), and a real-time PCR assay was performed to screen for the presence of the *tpi* gene *(59)*. A total of 10 μl of positive enrichment broth was plated onto chromID *C. difficile* agar (bioMérieux, Marcy l'Etoile, France) and incubated anaerobically at 37°C. Plates were checked for growth after 24 and 48 h. *C. difficile* colonies were confirmed by molecular techniques and tested for toxins (tcdA, tcdB, and cdtA) with real-time PCR assays *(25, 59)*. The template was prepared by suspending two or three colonies in 300 μl of 0.1% Tris-EDTA (Sigma Aldrich, Münich, Germany) and heating the solution for 15 min at 95°C. After short centrifugation (1 min at 14,000 × *g*), 5 μl of the supernatant was used as template with 12.5 μl of a commercial MasterMix (Agilent Technologies, Santa Clara, CA), 1 μl of the pUC 19 plasmid (1 fg/μl; Thermo Fisher Scientific, Waltham, MA), 500 μM concentrations of each primer, and 200 μM concentrations of each probe. *C. difficile* isolates were subcultured and stored in Cryobank tubes (Mast Diagnostica, Reinfeld, Germany) at −80°C. Reculturing for susceptibility testing was successful for 15 of the 24 isolates. Susceptibility testing was done using the agar disk diffusion test and the EUCAST disk diffusion method *(15)*. Common disk contents in veterinary and human medicine such as β-lactam antimicrobials, aminoglycosides, fluoroquinolones, trimethoprim-sulfamethoxazole, and tetracycline were used as listed in the EUCAST breakpoint and quality control tables *(15)*. Zone diameters were recorded as described in the EUCAST manual *(15)* after 48 h of anaerobic incubation on Mueller-Hinton agar with 5% sheep blood (Oxoid) at 37°C.

Laboratory testing: *C. botulinum*. A 10-g aliquot of each pooled fecal sample and 0.1 g of each dust sample were enriched in 90 ml and 10 ml, respectively, of TPGY broth, heated for 10 min at 60°C, and incubated for 96 h at 30°C in anaerobic jars (BD) with AnaeroGen sachets to create an anaerobic atmosphere.

DNA was extracted from 1-ml aliquots of each enrichment broth as described above. Real-time PCR assays for detection of toxin genes A, B, C, D, E, and F were performed using available protocols *(41, 59)*. Ten microliters of positive samples was streaked onto egg yolk agar (Heipha, Eppelheim, Germany) and blood agar (Oxoid) and incubated anaerobically for 72 h at 30°C. The presence of *C. botulinum* was confirmed by molecular techniques as described above.

Laboratory testing: *Y. enterocolitica*. A 10-g aliquot of each pooled fecal sample was placed into 90 ml of peptone sorbitol bile broth (Sigma-Aldrich) and incubated aerobically overnight at 25°C. DNA was extracted from a 1-ml aliquot of enrichment broth as described above and screened for the presence of the *ail* gene by real-time PCR *(40)*. A total of 10 μl of each positive enrichment broth was streaked onto CIN agar (Merck) with and without treatment with potassium hydroxide (EMD Chemicals, Millipore Corp., Billerica, MA) following ISO standard 10273 *(28)* and incubated aerobically for 48 h at 25°C. For treatment with potassium hydroxide, 0.5 ml of the enriched broth was vortexed with 4.5 ml of potassium hydroxide solution (0.25%), and 10 μl of the resultant solution was streaked onto CIN agar and incubated aerobically for 48 h at 25°C. Presumptive *Y. enterocolitica* colonies were identified by real-time PCR as described above.

Survey data. The farmers answered a short survey on the day of sampling to assess the use of antimicrobials in each tested group.

Statistical analysis. Statistical analysis was performed with Microsoft Excel (version 2010, Microsoft, Redmond, WA), and data were analyzed using chi-square tests (Epi Info, StatCalc version 6, Centers for Disease Control and Prevention, Atlanta, GA). When sample sizes were lower than 30, the Yates correction was applied. When any of the expected cell frequencies were less than 5, Fisher's exact test was used (Epi Info). Differences were considered significant at $P \leq 0.05$.

RESULTS

During a 1-year period from July 2011 to June 2012, 49 farms were screened for the presence of *C. difficile*, *C. botulinum*, and *Y. enterocolitica*. The results and prevalence rates are presented in Table 1 for dairy farms and in Table 2 for beef cattle farms.

***C. difficile*.** A total of 506 samples from 49 farms were analyzed. *C. difficile* genes were detected in at least one sample from 20 (40.8%) of the 49 farms: 18 (52.9%) of the

TABLE 1. *Prevalence of* C. difficile, C. botulinum, *and* Y. enterocolitica *on Bavarian dairy farms*

Sample type	Prevalence[a]		
	C. difficile	*C. botulinum*	*Y. enterocolitica*
Dairy cows			
Fecal	3/102 (2.9%) [1.0–8.3]	0/102 [0.0–3.6]	3/102 (2.9%) [1.0–8.3]
Dust	1/33 (3%) [0.5–15.3]	0/33 [0.0–10.4]	
Dairy calves			
Fecal	13/100 (13%) [7.8–21.0]	0/101 [0.0–3.7]	2/101 (2%) [0.5–6.9]
Dust	8/34 (23.5%) [12.4–40.0]	0/34 [0.0–10.2]	
Beef cattle			
Fecal	0/89 [0.0–4.1]	0/89 [0.0–4.1]	0/89 [0.0–4.1]
Dust	0/29 [0.0–11.7]	1/29 (3.5%) [0.6–17.2]	
Total	25/387 (6.5%) [4.4–9.4]	1/388 (0.3%) [0.05–1.5]	5/292 (1.7%) [0.7–4.0]

[a] Number of positive samples/number of samples tested (% of samples positive) [confidence interval].

34 dairy farms, and 2 (13.3%) of the 15 beef cattle farms. Dairy farms were significantly more likely to harbor *C. difficile* than were beef cattle farms ($P = 0.022$; relative risk [RR] = 3.97 [RR confidence interval, 1.05 to 14.99]). In total, *C. difficile* was detected in 29 (5.7%) of 506 samples: 25 (6.5%) of these samples were from dairy farms (Table 1), and 4 (3.4%) of these samples were from beef cattle farms (Table 2). On dairy farms, *C. difficile* was found in 4 (3%) fecal and dust samples obtained from the cow group and 21 (15.7%) fecal and dust samples from the calf group. In contrast, no samples were positive for *C. difficile* in the beef cattle group (Table 1). Fecal and dust samples obtained from the calf group were significantly more likely to contain *C. difficile* than were fecal samples from the dairy and beef cattle groups (fecal samples: $P = 0.008$, RR = 4.42 [1.3 to 15.0] and $P = 0.0004$; dust samples: $P = 0.027$, RR = 7.76 [$1.03 < \text{RR} < 58.71$] and $P = 0.006$). On beef cattle farms, *C. difficile* was detected in only the youngest group in two fecal samples (4.4%) and two dust samples (13.3%) (Table 2).

Twenty-four isolates were cultured from 29 PCR-positive samples. Toxin genes were identified in 17 (70.8%) of these 24 isolates from 13 farms (12 dairy farms and 1 beef cattle farm) (Table 3). Seven isolates had the $A^+B^+CDT^-$ toxigenic profile, seven had the $A^+B^-CDT^+$ profile, and three had the $A^+B^+CDT^+$ profile. Isolates from beef cattle farms all had the $A^+B^+CDT^-$ profile.

Antimicrobial susceptibility testing using the disk diffusion method with a panel of 19 antimicrobials was performed with 15 isolates. All isolates (100%) were susceptible to penicillin, ampicillin, florfenicol, erythromycin, tylosin, and trimethoprim-sulfamethoxazole. Four isolates (26.7%) were susceptible to oxacillin, and 13 isolates (86.7%) were susceptible to tetracycline. Resistance was detected for all isolates against enrofloxacin and aminoglycoside antibiotics, i.e., kanamycin, neomycin, and gentamicin. Fourteen isolates (93.3%) were resistant to apramycin, 9 isolates (60%) were resistant to cefalexin, and 14 (93.3%) were resistant to cefquinome.

C. botulinum. *C. botulinum* was detected in one dust sample (0.8% of all dust samples) from one dairy farm (2% of all samples) (Table 1). This isolate contained the gene coding for toxin B but could not be cultured. *C. botulinum* toxin genes were not detected in any fecal samples.

Y. enterocolitica. Three (6.1%) of the 49 farms provided samples that were positive for the *Y. enterocolitica ail* gene: 2 dairy farms and 1 beef cattle farm. *Y. enterocolitica* genes were identified in 6 (1.6%) of 382 fecal samples. Isolates from two samples, both from the same dairy farm, could be cultured on CIN agar. On dairy farms, 1.7% of the 292 fecal samples obtained from dairy cows and calves harbored *Y. enterocolitica* genes (Table 1). However, beef

TABLE 2. *Prevalence of* C. difficile, C. botulinum, *and* Y. enterocolitica *on Bavarian beef cattle farms*

Sample type	Prevalence[a]		
	C. difficile	*C. botulinum*	*Y. enterocolitica*
Youngest cattle			
Fecal	2/45 (4.4%) [1.2–14.8]	0/45 [0.0–7.9]	1/45 (2.2%) [0.4–11.6]
Dust	2/15 (13.3%) [3.7–37.9]	0/15 [0.0–20.4]	
Oldest cattle			
Fecal	0/45 [0.0–7.9]	0/45 [0.0–7.9]	0/45 [0.0–7.9]
Dust	0/14 [0.0–21.5]	0/14 [0.0–21.5]	
Total	4/119 (3.4%) [1.3–8.3]	0/119 [0.0–3.1]	1/90 (1.1%) [0.2–6.0]

[a] Number of positive samples/number of samples tested (% of samples positive) [confidence interval].

TABLE 3. *Toxigenic profiles of 24* C. difficile *isolates from Bavarian dairy and beef cattle farms*

Test animals	Prevalence[a]			
	$A^+B^+CDT^-$	$A^+B^-CDT^+$	$A^+B^+CDT^+$	No toxin genes
Dairy farms				
Cows	1/2 (50%)			1/2 (50%)
Calves	3/18 (16.7%)	7/18 (38.9%)	3/18 (16.7%)	5/18 (27.8%)
Beef cattle				
Beef cattle farms				
Youngest animals	3/4 (75%)			1/4 (25%)
Oldest animals				

[a] Number of positive samples/number of samples tested (% of samples positive).

cattle from dairy farms did not shed *Y. enterocolitica*. On beef cattle farms, 1 (1.1%) of 90 fecal samples was positive for *Y. enterocolitica* (Table 2).

Survey. The survey data revealed that on dairy farms 31 of 34 cow groups, 28 of 34 calf groups, and 8 of 30 beef cattle groups were treated with antimicrobials within the previous 6 months. On beef cattle farms, 14 of 15 of the youngest animal groups and 8 of 15 of the oldest animal groups had received antimicrobial treatment. However, these data did not reveal any correlation between the detection of *C. difficile* and the use of antimicrobials ($P = 1.0$ in all groups except the calf group, which had $P = 0.603$).

DISCUSSION

This study was designed to provide an overview of the occurrence of *C. difficile*, *C. botulinum*, and *Y. enterocolitica* among different ages of healthy cattle on different types of farms to assess the potential risk of transmitting bacteria via the food chain. The data indicate that *C. difficile* is rare on Bavarian farms, with a prevalence of 5.8%. *Y. enterocolitica* was less frequently detected (1.5% of all samples), and *C. botulinum* was found in only one dust sample (0.8% of all dust samples).

C. difficile. *C. difficile* was detected in 3.4% of 506 samples: 2.2% of fecal samples and 6.9% of dust samples on beef cattle farms. However, positive fecal and dust samples were detected only in the youngest group of cattle (Table 2). In previous studies, wide variation in the *C. difficile* shedding rate has been reported in beef cattle. In a U.S. study *(47)*, *C. difficile* was recovered from 12.9% of beef cattle at arrival on the farm and 1.2% of cattle before shipment to the slaughterhouse. In a Canadian study, *C. difficile* was recovered from 3.7% of cattle on arrival and 6.2% at mid-feeding *(11)*. Prevalence at the slaughterhouse was as high as 6.9% *(45)*. The variation in prevalences among these studies may be due to different sampling and culturing techniques. In the present study, pooled fecal samples were tested in contrast to individual rectal samples *(11, 45, 47)* and intestinal samples from slaughtered cattle *(45)*.

C. difficile prevalence in cows has been reported at 1.5 to 2.4% *(50, 55)*, which is consistent with the findings in our study in which 2.9% of fecal samples from the cow group contained *C. difficile*. However, in Austria *(27)* a slightly higher percentage of cows (4.5%) from the slaughterhouse carried *C. difficile*.

C. difficile was isolated from 13% of fecal samples obtained from the calf group in the present study, in agreement with finding from other studies. High *C. difficile* prevalence in calves has been reported: 9.5% in Slovenia as reported by Avbersek et al. *(2)*, 12.7% in a Swiss study *(50)*, and up to 51% in a Canadian study *(12)*. Calves from dairy farms were significantly more likely to carry *C. difficile* than were cows or beef cattle on these farms ($P = 0.008$, RR = 4.42 [1.3–15.0]; $P = 0.0004$). Various authors have hypothesized that age is a predisposing factor for calves acquiring *C. difficile (12, 48)*. The reason for this age predisposition is unclear; however, Costa et al. *(11, 12)* suggested that the intestinal flora of calves is not as well adapted as that of older animals, and therefore colonization with *C. difficile* might be easier. Other hypotheses have been proposed such as stress from birth or from overcrowding, an immature immune system, and the type of feeding and management system.

In various animal species and humans *(31, 32)*, the use of antimicrobials is a major risk factor for *C. difficile* infection because of their affect on the gut flora, which allows *C. difficile* to colonize. However, the survey data did not reveal a significant association between *C. difficile* detection rates and use of antimicrobials in all groups. Rodriguez-Palacios et al. *(48)* found that use of antimicrobials is a minor risk factor for calves for acquiring *C. difficile*. In the present study, *C. difficile* was found more often in younger than in older animals, although the cows (31 of 34 groups) were treated more often with antimicrobials than were the calves (28 of 34 groups). This finding suggests that a combination of age, use of antimicrobials, immune response of the calf, and type of feeding (milk versus roughage) can explain the fact that calves acquire *C. difficile* more frequently than do older cattle. However, only limited data on antimicrobial use were available in this study. To assess the use of antimicrobials as a risk factor, it would be necessary to conduct a study with larger sample sizes and more information concerning housing and feeding systems.

Antimicrobial susceptibility testing revealed that all *C. difficile* isolates were susceptible to antimicrobials commonly

used in veterinary medicine: penicillin, ampicillin, and florfenicol. However, resistance to other commonly used antimicrobials such as enrofloxacin, third generation cephalosporins, and aminoglycosides was variable, ranging from 60 to 93.3% of all isolates. In human medicine, *C. difficile* infections are mostly associated with prior use of antimicrobials. Therefore, it is necessary to determine which antimicrobials might select for *C. difficile*. In the present study, use of enrofloxacin, third generation cephalosporins, and aminoglycosides could exert selection pressure for *C. difficile*, as indicated by the susceptibility patterns found.

C. difficile has been found in beef cattle and food animals in general and in a variety of foods. In studies conducted in Northern America, *C. difficile* was found in 12 and 20.8% of ground beef in Canada *(49, 57)* and in 50% of ground beef in the United States *(54)*. In Europe, prevalence of *C. difficile* in ground beef ranged from 0% in Austria *(27)* and The Netherlands *(14)* to 1.9% in France *(8)* and 2.4% in Sweden *(56)*. The present study did not include meat products; however, no shedding of *C. difficile* by beef cattle prior to slaughter on both farm types was detected. Therefore, carcass contamination with *C. difficile* at the slaughterhouse seems to be very unlikely. *C. difficile* was detected in 13% of all fecal samples from calf groups, although most tested calves were younger than veal calves, which are 4 to 6 months of age at slaughter. The detection rate for *C. difficile* seems to decrease with the age of the animal, and therefore the expected prevalence of *C. difficile* in veal might also decrease. *C. difficile* thus could be transmitted via veal but with low prevalence, especially because veal is consumed far less often than is beef in Germany. In cows, *C. difficile* shedding rates were low; therefore, fecal contamination of milk and transmission of *C. difficile* into milk seems to be of minor importance.

These findings suggest that food animals and their products could be vectors of *C. difficile* for humans. However, it is unclear whether *C. difficile* infections in humans are foodborne *(47)*.

C. botulinum. *C. botulinum* was not detected in cattle and was detected only very rarely in their environment. One dust sample (0.3% of all samples) from a dairy farm harbored genes of *C. botulinum* type B toxin. In contrast, in a previous study in Germany 4% of fecal samples contained *C. botulinum (34)*. Notermans et al. *(43)* found that 13% of fecal samples from cattle were positive for toxin type B, whereas in cattle in Sweden the prevalence of *C. botulinum* type B toxin genes was reported as 73% and that of toxin types E and F was less than 5% *(13)*. The prevalence of *C. botulinum* in the present study was surprisingly low compared with the few existing data suggesting a higher prevalence for *C. botulinum* in German cattle and their environment. However, this variation within the same country could be due to differences in sample sizes; Klarmann *(34)* screened only 25 bovine fecal samples and found 1 sample that was positive for *C. botulinum* (a prevalence of 4%). In the two other studies conducted in other regions of Europe, prevalence rates differed depending on the country *(13, 34)*.

The finding of genes encoding botulinum neurotoxin type B in the dust sample is of potential concern because human foodborne botulism is usually caused by neurotoxin of types A, B, E, and F. In cattle botulism outbreaks, toxin types C and D were usually implicated; however, toxin types A and B have been described *(36)*. In 2010, seven outbreaks of *C. botulinum* infection were reported by European Union member states *(16)*. The most common food source in these outbreaks was pork meat followed by fish and fish products, vegetables, juice, and juice products. In the context of the very rare occurrence of *C. botulinum* in southern Germany, detection of genes encoding type B neurotoxin in one dust sample seems to be of minor importance for foodborne transmission. However, few studies concerning the prevalence of *C. botulinum* in healthy cattle and the environment have been conducted to assess potential foodborne transmission of this pathogen.

Y. enterocolitica. *Y. enterocolitica* was detected on 3 (6.1%) of the 49 farms, with a prevalence of 1.6% of fecal samples from cattle, consistent with findings in previous studies. Prevalence can range from 0% as reported by Bailey et al. *(3)* and Bucher et al. *(9)*, to 6.3 and 4.5% as reported in two British studies *(39, 42)*, and up to 14.3% *(7)*. In the present study, *Y. enterocolitica* was detected in cattle but at a very low prevalence. Thus, beef or veal could be a vector for transmitting *Y. enterocolitica*, but this route probably is not of major importance. To further assess beef as a vector for *Y. enterocolitica*, studies with larger sample sizes are needed. Other sources of foodborne *Y. enterocolitica* have been described in the United States, where a case of human yersiniosis was reported from drinking pasteurized milk *(10)* and *Y. enterocolitica* was found in bulk tank milk *(29)*. However, in a German study *Y. enterocolitica* was not detected in raw milk samples *(40)*.Various sources for foodborne yersiniosis have been described with different rates of occurrence. However, because only a small number of samples were positive for *Y. enterocolitica* in the present study, cattle may be only a minor source of human yersiniosis in Bavaria.

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IV. DISCUSSION

1. ESBL *E. coli*

The study about the prevalence of ESBL *E. coli* on Bavarian dairy and beef cattle farms was conducted within the RESET joint research project (www.reset-verbund.de) including research projects in humans, different animal species, the environment and food across Germany. This interdisciplinary joint research project has been funded by the Federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung). The aim of this research project is to assess the effect of ESBL *E. coli*, their origin, way of transmission and the risk for humans being exposed to them. On the basis of this assessment possible recommendations can be made concerning the control of resistant bacteria on a rational basis. These discussions are necessary to determine potential risks of the spread of ESBL *E. coli* and to further implement actions if needed, particularly on a legal level, such as improving control of antimicrobial usage, restricted use of single agents, etc.

To prevent the occurrence and spread of resistant bacteria guidelines on prudent use of antimicrobials exist in veterinary medicine in contrast to human medicine. The “guidelines for the careful handling of antibacterial veterinary drugs” (“Leitlinien für den sorgfältigen Umgang mit antibakteriell wirksamen Tierarzneimitteln”) (BTK, 2010), have been developed by the working committee on veterinary drugs (Arbeitsgruppe Tierarzneimittel der Länderarbeitsgemeinschaft Verbraucherschutz, Bundestierärztekammer) and provide information on antimicrobial usage specially conceptualized for certain animal species. These guidelines give advice on the indication of certain drugs as well as dosage and treatment length, trying to minimize consumption in veterinary medicine, with the goal of decreasing resistant bacteria.

The study showed that ESBL *E. coli* are commonly found on Bavarian dairy and beef cattle farms: They could be detected on 86.7% of all farms with a

prevalence of 32.8% of all samples. A total of 28 of 30 mixed cattle farms and 11 of 15 beef cattle farms harbored at least one sample that contained ESBL *E. coli.* The prevalence of ESBL *E. coli* positive fecal samples on mixed cattle farms was 39.6% compared to 18.9% on beef cattle farms, which is consistent with other European studies: In Switzerland (GESER et al., 2012) 13.7% of bovine fecal samples from the slaughterhouse harbored ESBL *E. coli*, as well as 10.2% in Denmark (DANMAP, 2012), 4.1% in France (MADEC et al., 2008) and up to 44.3% in the UK (HORTON et al., 2011). The first two studies screened bovine fecal samples from the slaughterhouse, whereas the British study screened bovine fecal samples from animals on farms and the French study screened fecal samples from both origins. Prevalence rates vary in European countries, which can be due to different methodologies and sample sizes that make comparison of data difficult. However, ESBL *E. coli* are widely spread through-out different countries.

Moreover, on mixed farms fecal samples and boot swabs obtained from the calf group were significantly more likely to harbor ESBL *E. coli* than fecal samples and boot swabs from the dairy and beef cattle group. This finding is consistent with other studies (WATSON et al., 2012), where CTX-M prevalence in calves was up to 98.7% within 21 days of birth. This study further showed a decline of prevalence with increasing age of calves: At 117 days of age only 10% of the calves shed CTX-M *E. coli*. The authors suggested that diet could be one of the reasons why younger calves shed more ESBL *E. coli* than older ones: Pre-weaned calves possess a different gut flora than older ones due to a shift in bacteria with less gram negative bacteria. Moreover, these authors proposed an age related immune effect which may reduce fecal shedding of ESBL *E. coli*. Other studies (KHACHATRYAN et al., 2004; BERGE et al., 2006; SINGER et al., 2008) showed as well, that the age of a calf is a strong predictor of antimicrobial resistance: Especially pre-weaned calves seem to be a reservoir for resistant bacteria. However, the calves in the study from KHACHATRYAN et al. (2004) and some of the calves from the study conducted by BERGE et al. (2006) were fed treated milk replacer. BERGE et al. (2006) stated that the use of antimicrobials is a dominant selective influence for resistant bacteria which is in consistency with

our findings. This may be one of the main reasons why calves are more likely to shed ESBL *E. coli*. They are more prone to develop diseases as diarrhea, pneumonia or navel infections than older animals, potentially leading to systemic treatment with antimicrobials. The treatment does not have to include the use of β-lactam antimicrobials as other classes of antimicrobials can co-select for ESBL resistance genes in bacteria as well. Resistance determinants against cephalosporins, aminoglycosides, fluoroquinolones, tetracycline, sulfonamides, as well as disinfectants are often situated on the same plasmid and therefore, can be co-selected (JACOBY & SUTTON, 1991). These plasmids can also code for genes mediating heavy metal tolerance, resistance to ultraviolet light, virulence and metabolic functions (BARBOSA & LEVY, 2000). Selection for these plasmid-encoded characteristics may also contribute to the persistence of plasmid-borne antimicrobial resistance, even in the absence of antimicrobial usage. This means, that even without the use of beta-lactam antimicrobials or antimicrobials in general, ESBL resistance genes can be co-selected and, therefore, can persist in bacteria.

Furthermore, our study indicated that feeding waste milk and, therefore, low concentrations of antimicrobials, may pose a potential selective pressure and may be an advantage for bacteria with resistance to antimicrobials: Calves being fed waste milk harbored more ESBL-producing *E. coli*-positive fecal samples than calves fed otherwise. This difference, however, was not significant ($p=0.055$, median for calves without waste milk, 0.33; median for calves with waste milk, 0.83). Similar hypotheses were stated by BERGE et al. (2005).

Moreover, BERGE et al. (2005) detected heifers shedding ESBL *E. coli* even in the absence of antimicrobial therapy. One hypothesis for this finding was that individual animal treatment may provide enough selective pressure to maintain a resistant gene pool in the farm-level bacteria population and, therefore, may spread to not-treated animals.

In general samples from the beef cattle group of mixed farms and samples obtained from beef cattle farms were less likely to harbor ESBL *E. coli* than samples from all other groups. This can be partly due to fewer treatments with antimicrobials. Most beef cattle tested in this study were four months and older.

Older calves and cattle are less likely to suffer from infectious diseases and, therefore, may be treated less often. Furthermore, older calves possess a different gut flora because of their diet and a more “aged” immune system which may favor the colonization of a more susceptible set of strains (BERGE et al., 2005; SINGER et al., 2008). These factors may lead to fewer resistant bacteria in this animal group.

In the control group ESBL *E. coli* could only be detected in boot swabs but not in fecal samples. Moreover, much fewer control farms (3 of 10, 30%) were positive for ESBL *E. coli* in contrast to mixed farms and beef cattle farms, where at least one sample was positive on 28 out of 30 (93.3%) and 11 out of 15 farms (73.3%) respectively. This suggests that ESBL *E. coli* can be found more often on farms that use antimicrobials as control farms did not use antimicrobials for the last six months, whereas mixed and beef cattle farms did use them, except for one beef cattle farm. Antimicrobials have not been used in every group on mixed and beef cattle farms, therefore, statistical analysis was performed for each group to detect a possible difference in prevalence among treated and untreated groups. This analysis, though, only revealed a statistical significance for the calf group on mixed farms: Calves being treated with antimicrobials shed significantly more often ESBL *E. coli* than untreated calves ($p=0.041$). For all other groups on mixed and beef cattle farms no statistical relation could be determined. This could be due to the small number of farms tested and the resulting sample size. With more farms being part of the study and the control group the effect of use of antimicrobials on ESBL *E. coli* prevalence rate would probably be more obvious. However, the data indicate a potential relation between the use of antimicrobials on farms and the detection rate of ESBL *E. coli* on these farms.

The study also showed that ESBL *E. coli* isolates mostly harbored genes encoding $bla_{\text{CTX-M}}$, particularly of the CTX-M-1 group. Resistance genes belonging to group CTX-M-2 and -9 could be found as well. ESBL resistance genes were distributed equally among mixed dairy and beef cattle farms and exclusively beef cattle farms. However, AmpC resistance on mixed farms was solely due to a mutation of the promoter region in contrast to beef cattle farms where plasmid-mediated ampC resistance genes as CMY-2 and FOX could be

found, too. In animals and humans the predominantly found resistance genes can differ: CTX-M-15 is the predominant *bla* found in humans, but only occasionally found in animals. CTX-M-1, however, is equally detected among humans and animals. Moreover, plasmid-mediated ampC resistance genes, as found on beef cattle farms, play an important role in the transmission of resistance genes. Many resistance genes, as ESBL and ampC, are plasmid-mediated and can spread more easily via horizontal gene transfer than chromosomal ones. This knowledge about predominantly found resistance genes in animals, humans and food and their location on either the chromosome or plasmids is of main importance when assessing risk factors and transmission of resistant bacteria.

The Federal Ministry of Food, Agriculture and Consumer Protection (Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz, BMELV) demands a considerably reduction of antimicrobials in veterinary medicine combined with tighter controls and more detailed records, similar to approaches made in Denmark and The Netherlands. An amendment of the German Medicinal Products Act (AMG) was passed in 2013. The objective of this amendment is to reduce use of antimicrobials in livestock and to get transparency of the amount of drugs used via a database that records its usage. The use of antimicrobials above average can be detected by the authorities via this database and interventions and actions can be suggested to reduce their consumption. As part of the interventions animal husbandry and vaccination protocols need to be evaluated as a decrease of antimicrobial consumption also depends on the condition animals are kept under. This will reduce the number of infections and, therefore, the need to use antimicrobials (FVE, 2011).

To be able to rationally discuss implementations of legal actions as minimizing use of antimicrobials, detailed epidemiologic studies in human and veterinary medicine including animal production as well as pet animals are needed. This type of studies provides basic knowledge about the occurrence and spread of resistant bacteria and can show where improvements are needed. Unfortunately, there is still a paucity of studies concerning prevalence rates on a regional or country-wide scale in Europe.

2. *C. difficile*, *C. botulinum* and *Y. enterocolitica*

C. difficile, *C. botulinum* and *Y. enterocolitica* can potentially be transmitted via the food chain leading to foodborne zoonosis. The EUROPEAN COUNCIL (1992) defined zoonosis as "[…] any disease and/or infection which is likely to be naturally transmitted from animals to men" either via direct contact or contaminated food. To prevent zoonoses, it is important to identify which animals and food are the main sources of infections. Therefore, data collection and analysis is essential and supports prevention and reduction of transmission of pathogens via animals or food.

There are only limited data available concerning prevalence rates of *C. difficile*, *C. botulinum* and *Y. enterocolitica* in healthy cattle, especially concerning the last two pathogens. Moreover, data about prevalence rates of *C. botulinum* and *Y. enterocolitica* are possibly outdated as most studies were conducted more than a decade ago. Therefore, we assessed prevalence rates of *C. difficile*, *C. botulinum* and *Y. enterocolitica* in healthy dairy and beef cattle in Bavaria. This study revealed that *C. difficile* and *Y. enterocolitica* could rarely be found in healthy cattle with prevalence rates of 5.7% and 1.6% respectively. In contrast to previous studies *C. botulinum* could not be detected in fecal samples, but very rarely in dust samples (0.8%) from the cattle environment. Other studies detected *C. botulinum* in fecal samples, with prevalence rates ranging from 4% (KLARMANN, 1989) up to 73% in cattle (DAHLENBORG et al., 2003). This variation may be due to different methodologies, but moreover, to regional differences: In countries surrounded by water, particularly the Northern countries in the EU, *C. botulinum* spores can commonly be found in the water and soil (SMITH & YOUNG, 1980; HIELM S et al., 1998) representing a potential reservoir for animals. One of these studies was conducted by Klarmann et al. in Germany in 1989 and showed different results. These variations of findings in the same country could be due to changes of prevalence in the course of time, but could also be due to sample sizes: Klarmann et al. screened 25 bovine fecal samples for the occurrence of *C. botulinum* yielding one positive sample (4%).

C. difficile could not be detected in beef cattle prior to slaughter as well as *Y. enterocolitica*. Moreover, both pathogens were only rarely found in lactating dairy cows with prevalences of 2.9% each. Contamination of meat could occur during the slaughter process by carcass coming into contact with intestinal contents and contamination of milk could occur during the milking process by improper hygiene management. As fecal samples obtained from beef cattle prior to slaughter were negative for *C. difficile* and *Y. enterocolitica* and these pathogens could only rarely be found in lactating dairy cows this suggests that these pathogens as well as *C. botulinum*, which could not be detected in bovine fecal samples at all, are unlikely transmitted via milk and meat.

3. Conclusion

The present study shows that ESBL-producing *E. coli* can commonly be found on Bavarian dairy and beef cattle farms, though, at different prevalence rates depending on the farm type. On mixed farms calves were more likely to shed ESBL *E. coli* followed by cows and beef cattle. It indicates that resistant bacteria are present in groups where antimicrobials have been used and suggests that calves may be a reservoir for these bacteria. The results were consistent with other European studies revealing that ESBL *E. coli* are widely spread all over Europe as well as the rest of the world.

The data further suggest that dairy and beef cattle seem to be a minor source for transmitting *C. difficile*, *C. botulinum* and *Y. enterocolitica* to humans via the food chain. However, this data does not necessarily have to apply for the whole of Germany as this study provides data for only a circumscribed area (southern Bavaria) with characteristically small animal plants.

There is a paucity of studies that show a countrywide prevalence in Germany and other countries. Additional nationwide surveys would be necessary to assess prevalence rates for resistant bacteria, as well as for potentially foodborne pathogens, to determine the risk of transmission from animals and their products to humans. This is necessary to provide a solid basis for taking effective and timely risk management decisions to ensure public health.

V. SUMMARY

To ensure food safety the idea of an integrated approach of surveillance “from farm to fork“ was established to enable the risk assessment and risk management on every level of the food chain. Pathogen prevalence monitoring is an essential part in detecting and evaluating a potential transmission of pathogens, particularly in primary production where transmission can occur via animals and/or their products. Furthermore, consumers are concerned about increasing reports of resistant bacteria, as a growing number of extended-spectrum β-lactamases have been identified in *Enterobacteriaceae* during the last few years. They were not only detected in humans, but also in a broad range of animal species and also in food.

Therefore, the present study was conducted to determine first, the prevalence of ESBL *E. coli* and second, the prevalences of *C. difficile, C. botulinum* and *Y. enterocolitica* on dairy and beef cattle farms in the southern part of Bavaria. Fecal samples, boot swabs and dust samples were collected in a one-year period from summer 2011 to summer 2012.

ESBL *E.coli* could be detected by selective culturing after enrichment in a total of 196 of 598 samples (32.8%) and originated from 39 (86.7%) of 45 farms. Samples obtained from mixed dairy and beef cattle farms were significantly more likely to harbor ESBL *E. coli* than samples from beef cattle farms (percentage of ESBL *E. coli* in fecal samples from mixed farms: 39.6%, in fecal samples from beef cattle farms: 18.9%; in boot swabs from mixed farms: 51.1%, in boot swabs from beef cattle farms: 25.8%; in dust samples from mixed farms: 19.3%, in dust samples from beef cattle farms: 3.5%). The highest rates of ESBL *E. coli* on mixed farms could be detected in calves with 56.2% of fecal samples being positive for ESBL *E. coli* followed by cows (41.1% of fecal samples) and beef cattle (21.4% of fecal samples). Furthermore, a total of 183 isolates (93.4%) of 196 ESBL *E. coli* positive strains harbored CTX-M genes, CTX-M group 1 being the most frequently found group. Forty six additional isolates contained *ampC* genes and five of the 46 isolates expressed a $bla_{\text{CMY-2}}$ gene.

C. difficile genes were detected in 29 of 506 samples (5.7%), where 25 samples originated from dairy farms and four from beef cattle farms. Toxin genes were identified in 17 samples showing the toxigenic profiles $A^+B^+CDT^-$, $A^+B^-CDT^+$, and $A^+B^+CDT^+$. *C. botulinum* toxin genes could not be detected in fecal samples from cattle, but in one dust sample (0.8%) harboring toxin gene B. *Y. enterocolitica* genes were found in six of 382 fecal samples (1.6%) from three dairy farms and one beef cattle farm.

This study reveals that ESBL *E. coli* are commonly found on Bavarian dairy and beef cattle farms, whereas *C. difficile* and *Y. enterocolitica* can only rarely be found. In contrast to previous studies *C. botulinum* could not be detected in fecal samples, but sporadically in dust samples from the cattle environment. These results lead to the conclusion that ESBL-producing bacteria can potentially be transferred to humans via animals or their products. However, there is still a paucity of studies giving evidence for such a transfer. Furthermore, the data suggest that, at least in Bavaria, cattle and their products may be a minor reservoir for transmitting *C. difficile, C. botulinum* and *Y. enterocolitica* to humans.

VI. ZUSAMMENFASSUNG

Prävalenz von Extended- Spectrum β- Laktamase-produzierenden *Escherichia coli*, toxinogenen *Clostridium spp.* und *Yersinia enterocolitica* in bayerischen Milch- und Mastrinderbeständen

Um die Sicherheit der Lebensmittel zu gewährleisten wurde die Idee eines integrierten Ansatzes der Überwachung „from farm to fork“ aufgebaut, um auf jeder Ebene der Lebensmittelkette die Risikobewertung und das Risikomanagement zu ermöglichen. Das Prävalenzmonitoring von Pathogenen ist ein grundlegender Teil beim Nachweis und der Beurteilung einer potentiellen Übertragung von Krankheitserregern, vor allem in der Primärproduktion, wo die Übertragung über Tiere und/oder deren Produkte erfolgen kann. Weiterhin sind Konsumenten über zunehmende Berichte von resistenten Bakterien beunruhigt, da eine steigende Anzahl von Extended-Spectrum β-Laktamasen in *Enterobacteriaceae* in den letzten Jahren nicht nur bei Menschen, sondern auch in einer Vielzahl von Tierarten und in Lebensmitteln nachgewiesen wurden.

Die vorliegende Studie wurde daher durchgeführt, um zum Einen die Prävalenz von ESBL *E. coli* und zum Anderen die Prävalenzen von *C. difficile, C. botulinum* und *Y. enterocolitica* in Milch- und Mastrinderbetrieben in Südbayern zu ermitteln. Während eines Jahres, von Sommer 2011 bis Sommer 2012, wurden Kotproben, Sockentupfer, und Staubproben gesammelt.

ESBL *E. coli* konnten nach Anreicherung auf selektiven Nährböden in 196 von 598 (32,8%) Proben angezüchtet werden und stammten von 39 von insgesamt 45 (86,7%) Betrieben. Proben, die von kombinierten Milch- und Mastrinderbetrieben stammten, enthielten signifikant häufiger ESBL *E.coli* als Proben von reinen Mastrinderbetrieben (Prozent ESBL *E. coli* in Kotproben von Kombibetrieben: 39,6%, in Kotproben von Mastbetrieben: 18,9%; in

Sockentupfern von Kombibetrieben: 51,1%, in Sockentupfern von Mastbetrieben: 25,8%; in Staubproben von Kombibetrieben: 19,3%, in Staubproben von Mastbetrieben: 3,5%). Der größte Anteil an ESBL *E. coli* auf Kombibetrieben konnte bei Kälbern gefunden werden, mit 56,2% ESBL *E. coli* positiven Kotproben, gefolgt von den Kühen (41,1% der Kotproben) und Mastrindern (21,4% der Kotproben). Weiterhin enthielten 183 von 196 (93,4%) ESBL *E. coli* positiven Stämme CTX-M Gene, wobei CTX-M Gruppe 1 die am häufigsten vorgefundene Gruppe war. Weitere 46 Isolate enthielten *ampC* Gene, wobei fünf der 46 Isolate ein bla_{CMY-2} Gen exprimierten.

Gene für *C. difficile* wurden in 29 von 506 Proben (5,7%) gefunden, wobei 25 Proben von Milch- und vier von Mastrinderbetrieben stammten. Toxingene wurden in 17 Proben ermittelt und zeigten die Toxingenprofile $A^+B^+CDT^-$, $A^+B^-CDT^+$, und $A^+B^+CDT^+$. *C. botulinum* Toxingene konnten in den Rinderkotproben nicht nachgewiesen werden, jedoch in einer Staubprobe (0,8%), die Toxingen B enthielt. Gene von *Y. enterocolitica* wurden in sechs von 382 Kotproben (1,6%) von drei Milch- und einem Mastrinderbetrieb gefunden.

Diese Studie zeigt, dass ESBL *E. coli* häufig auf bayerischen Milch- und Mastrinderbeständen gefunden wird, wohingegen *C. difficile* und *Y. enterocolitica* nur selten nachgewiesen werden können. Im Gegensatz zu vorangegangenen Studien konnte *C. botulinum* nicht in Kotproben nachgewiesen werden, aber vereinzelt in Staubproben aus der Umgebung der Rinder. Diese Ergebnisse führen zu dem Schluss, dass ESBL-Bildner potentiell über Tiere oder deren Produkte auf den Menschen übertragen werden können. Dennoch gibt es einen Mangel an Studien, die solch einen Transfer beweisen. Weiterhin weisen die Daten darauf hin, dass zumindest in Bayern Rinder und deren Produkte ein unbedeutendes Reservoir für die Übertragung von *C. difficile, C. botulinum* und *Y. enterocolitica* auf Menschen zu sein scheinen.

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